

**INFLAMMATION AND HAEMOSTASIS IN THE DEVELOPMENT AND
PROGRESSION OF PERIPHERAL ATHEROSCLEROTIC DISEASE**

by

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DECLARATION

Thesis: Inflammation and haemostasis in the development and progression of peripheral atherosclerotic disease.

I, Ioanna Tzoulaki hereby declare that I am the sole author of this thesis. It has not been submitted for any other degree, and all sources of information have been acknowledged. I conducted all aspects of the research except for the baseline examination of the Edinburgh Artery Study, the follow up of the sample until the 15 years and the laboratory assays.

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ABSTRACT

Peripheral arterial disease (PAD) defines atherosclerotic disease of the arteries to the legs. PAD begins early in life and remains asymptomatic over long periods. The ankle brachial index (ABI) is an important diagnostic test which can identify asymptomatic individuals and serve as a good marker of the underlying peripheral and systemic atherosclerosis. Recent advances in vascular biology proposed a role of inflammatory and haemostatic mechanisms in atherosclerotic disease. Although inflammatory and haemostatic markers have been associated with coronary atherosclerosis in large scale epidemiological studies their role in PAD development is not well established and for many markers unknown. Also, their relationship with the progression of early asymptomatic disease has not been studied before.

The aim of this thesis was to examine 12 markers of inflammation and haemostasis in relation to peripheral atherosclerotic progression and incident PAD. The Edinburgh Artery Study was used for this analysis. This is a population based cohort study of 1,592 men and women recruited in 1987. ABI was measured at baseline and at two follow up examinations which were conducted after 5 and after 12 years. Also, subjects were followed up for cardiovascular events for 17 years. Conventional cardiovascular risk factors, C-reactive protein (CRP), interleukin-6 (IL-6), intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, fibrinogen, D-dimer, tissue plasminogen activator (t-PA), vonWillebrand factor (vWF), factor VII,

fibrinopeptide A (FpA) and prothrombin fragments 1+2 (F1+2) were measured at baseline.

Valid ABI measurements were available for 1,582 subjects at baseline, for 1,081 subjects at the 5 year follow up and for 816 subjects at the 12 year follow up. The population showed a progression in atherosclerotic disease assessed by the mean ABI decline over time. The mean change in ABI was -0.04 (0.18) after 5 years and -0.06 (0.19) after 12 years. From inflammatory markers, CRP ($p < 0.01$), IL-6 ($p < 0.001$) and ICAM-1 ($p < 0.01$) were associated with atherosclerotic progression after 12 years, independently of baseline ABI and of conventional cardiovascular risk factors. Also, from haemostatic markers, fibrinogen ($p = 0.05$) and D-dimer ($p \leq 0.05$) were significantly associated with atherosclerotic progression independently of baseline ABI and cardiovascular risk factors. Moreover, subjects with higher levels of both D-dimer and IL-6 at baseline had the greatest ABI decline. Also, IL-6 showed the stronger independent effect on atherosclerotic progression and retained statistical significance after adjustments for all inflammatory markers and for fibrinogen and D-dimer.

Approximately 26% of the baseline population developed at least one event of major CVD and 14% of the baseline population developed symptomatic PAD after 17 years of follow up. Inflammatory markers, CRP and IL-6 showed modest associations with PAD which lost statistical significance in the multivariable model. On the other hand, these markers were associated with incident major CVD with hazard ratios (95% CI) 1.6 (1.2, 2.3) and 1.8 (1.3, 2.6) respectively (top vs. bottom tertile) in the multivariable model.

ICAM-1 showed weak associations with incident CVD, however, was significantly associated with PAD with hazard ratio (95% CI) 1.8 (1.2, 2.7) (top vs. bottom tertile) after adjustments for cardiovascular risk factors and CVD at baseline. Haemostatic markers, fibrinogen and D-dimer were associated with 2.2 (95% CI: 1.5, 3.2) and 1.7 (1.2, 2.6) increase in the risk of PAD development and 1.8 (1.3, 2.3) and 1.6 (1.2, 2.1) increase in the risk of CVD independently of cardiovascular risk factors and history of CVD at baseline, respectively.

This analysis showed a major role of inflammatory markers, CRP, IL-6 and ICAM-1 in atherosclerotic development and progression. In addition, fibrinogen and D-dimer, but not other haemostatic factors, were associated with progressive and incident peripheral atherosclerosis. Since D-dimer and fibrinogen are acute phase reactants, these data support the hypothesis that inflammation is more related to atherosclerosis than is hypercoagulation. Most importantly, the majority of the reported associations were not explained by increased levels of cardiovascular risk factors or pre-existing clinical or subclinical arterial disease. Thus these markers are more likely to have a causal than a consequential role in atherosclerotic disease.

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Chapter one

1 Peripheral arterial disease

1.1 Introduction

This chapter describes the epidemiology of peripheral arterial disease (PAD). The prevalence and incidence along with the natural history of the disease as evaluated in epidemiologic research are discussed. In addition, the established risk factors for the development and progression of the disease are summarized. Finally, the different diagnostic tests for the diagnosis of PAD are described with particular focus on the ankle brachial index (ABI); the most commonly used non-invasive test for PAD.

1.2 Definition of PAD

Atherosclerosis is a progressive disease of the large arteries characterized by the accumulation of lipids and fibrous elements in the arterial wall (Lusis 2000). At its earliest state consists of a 'fatty streak' which consists of subendothelial accumulations of cholesterol-engorged macrophages known as foam cells. The fatty streaks are not clinically important but are the precursors of more serious lesions and eventually of the atherosclerotic plaque. Plaques often become gradually more complex with calcification, ulceration and haemorrhage. Although, these lesion can grow large and eventually block blood flow, the most important and common complication is the rupture of the atherosclerotic plaque and the formation of the thrombus (Lusis 2000).

Atherosclerosis can affect several arterial beds. The term PAD refers to atherosclerotic disease that obstructs the blood supply to the lower limbs. Considerable confusion exists concerning this term because some investigators include carotid, mesenteric, renal and upper extremity disease in the definition of PAD. The term peripheral vascular disease (PVD) has less specificity since for many researchers this includes venous as well as arterial disease. Other terms have been used including peripheral arterial occlusive disease (PAOD), arteriosclerosis obliterans (ASO) and lower extremity arterial disease (LEAD). In this thesis, the term PAD is used solely to describe atherosclerotic disease in the arteries to the legs.

PAD starts for many individuals early in life and remains asymptomatic for a long period of time. The disease often has clinical symptoms when it is relatively advanced. The most common symptom is intermittent claudication (IC), a cramping pain in the legs which is induced by exercise and relieved by rest. When PAD progresses to severe impairment of blood flow to the limb due to arterial stenosis and occlusion, an individual is considered to have critical limb ischemia (CLI). CLI is often characterized by persistent rest pain which becomes worse when the legs are elevated e.g. in bed at night. People diagnosed with CLI may also present with gangrene and ulceration in their legs. Finally, if the disease progresses further, patients might have to undergo surgical treatment and leg amputation.

1.3 Prevalence and incidence of PAD

PAD is a relatively common condition which affects many adults worldwide (Hirsch et al 2006). The prevalence and incidence of PAD in populations has been measured in epidemiological studies that have used various definitions of PAD. Some use IC to define PAD whereas others use non-invasive tests like an abnormal ABI to measure asymptomatic forms of the disease. Few reports focus on the later disease stages of CLI. In general, the prevalence of PAD varies with the age of the cohort studied, the risk factor profile of the cohort, and the diagnostic test used to measure the disease. An overview of population studies on the prevalence and incidence of lower extremity atherosclerosis is given here.

1.3.1 Intermittent claudication

IC as a symptomatic expression of PAD defines a subset of the total population with the disease. Epidemiological studies have assessed the prevalence of IC mainly by means of the World Health Organization (WHO) IC questionnaire (Appendix I). Overall, the estimated prevalence of claudication assessed by an IC questionnaire ranges from 0.4 to 14.4% (Dormandy et al 1989). For example, in the Limburg study of 3,654 individuals aged 40-79 years the prevalence of IC using the Rose criteria was 1.4% (Stoffers et al 1991). Similarly, the Whitehall study on over 18,000 individuals (40-69 years old) estimated the prevalence of IC at 1.8% (Smith et al 1990). The Edinburgh Artery Study reported a considerable higher prevalence of 4.5% for older people aged 55-74 years. Table 1 lists the aforementioned and other large scale studies that have estimated the

prevalence of IC with the WHO IC questionnaire in population samples. As Table 1 shows, the prevalence of IC varied across studies and overall increased with age. Some evidence that IC is slightly more common in males than females is also presented. However, gender differences have not been observed in other studies. In the Edinburgh Artery Study (Fowkes et al 1991) of people aged 55-74 years the prevalence of IC was the same (4.6%) in men and women. In support of these findings, Reunanen (Reunanen et al 1982) reported 2.1% prevalence in men and 1.8% in women and the age adjusted prevalence was almost equal. These findings are keeping with an equilibration with frequency of cardiovascular disease (CVD) between men and women at older ages.

There are fewer reports on the incidence of PAD in populations. In the Framingham Heart Study, incidence of PAD was based on IC symptoms in 2,336 men and 2,873 women aged 29-62 years. The annual incidence was estimated to be 6 per 10,000 men and 3 per 10,000 women for those aged 30-44 and 61 per 10,000 men and 54 per 10,000 women for those aged between 65-74 years (Kannel et al 1970). In the Edinburgh Artery Study of 1,529 subjects aged 55-74 years old the 5-year cumulative incidence was estimated at 9% when PAD was diagnosed by means of the WHO IC questionnaire (Leng et al 1996a). In another study in Israel of 8,343 men aged 40-65 years, after 21 years, the cumulative incidence was 43.1 per 1,000 population (Bowlin et al 1997). On the other hand, the Quebec Cardiovascular study (Dagenais et al 1991) estimated the incidence of PAD at 41 per 10,000 population per year whereas the US Physicians Health Study (Camargo et al 1997) reported 433 PAD events among 22,071 healthy males during 11 years of follow up. These results demonstrate that like prevalence the

incidence in populations varies greatly depending on age structure and characteristics of the populations as well as the epidemiological methods used. However, most studies show that the incidence increased substantially with age.

Table 1 Prevalence of intermittent claudication (IC) in different population studies assessed by means of the WHO/ IC questionnaire

Reference	N	Population	Location	Age (years)	IC (%)
Bothig et al 1976	-	M	Moscow	50-64	6.9
		M	Berlin	50-64	3.4
Hughson et al 1978	162	MW	England	45-69	2.2
Schroll & Munck 1981	666	MW	Denmark	>60	1.8
Reunanen et al 1982	5,738	M	Finland	30-59	2.1
	5,224	F		30-59	1.8
Criqui et al 1985	613	MW	USA	38-82	2.2
Gofin et al 1987	1,036	M	Jerusalem	40-60	1.3
	556	W			1.8
Smith et al 1990	18,388	MW	Scotland	40-64	1.8
Stoffers et al 1990	3,654	MF	Holland	45-54	0.6
				55-64	2.5
				65-74	8.8
Fowkes et al 1991	1,592	MW	Scotland	55-74	4.6
Dewhurst et al 1991	259	MW	England	65-95	5
Newman et al 1993	2,214	M	USA	65-85	2.0
	2,870	W			
Skau et al 1993	2,748	MW	Sweden	50-89	4.1
Mittelmark et al 1993	5,201	M	USA	>65	3.0
Aronow et al 1994	1,160	M	USA	80	32
	2,464	W		81	26
Bowlin et al 1994	10,059	M	Israel	40-65	2.7

Table 1 cont.					
Bainton et al 1994	2,055	M	Scotland	60-64	2.9
Wilt et al 1996	4,159	MW	USA	-	8.5
Zheng et al 1997	1,553	M [†]	USA	45-64	0.6
	2,518	W [†]			0.5
	5,207	M [‡]		45-64	1.1
	5,828	W [‡]			0.6
Meijer et al 1998	7,715	MW	Holland	>55	1.6
Ness et al 2000	467	M	USA	80	20
	1,444	W		81	13
Murabito et al 2002	1,554	M	USA	>40	3.9
	1,759	W			3.3
Brevetti et al 2004	4,352	MW	Italy	40-80	1.6
Diehm et al 2004	6,821	MW	Germany	>65	2.8
M: men, W: women					
† African-American, ‡ White					

1.3.2 Asymptomatic disease

The prevalence of asymptomatic disease can only be estimated by non-invasive diagnostic techniques. Therefore, the estimated prevalence is greatly dependent on the actual measurement technique used in each study. In general, the prevalence of asymptomatic disease is higher than that estimated on the basis of IC symptoms and ranges between 0.9% and 22% with the ratio of symptomatic to asymptomatic ranging between 1:0.9 and 1:6.0 (Dormandy et al 2000). Pioneer research by Hiatt et al. (Hiatt et al 1995) showed that for every individual with IC there are another 3 with asymptomatic disease causing a 50% or greater stenosis of the arteries supplying the legs. The predominance of asymptomatic patients was also demonstrated by Stoffers (Stoffers et al 1996) who investigated the prevalence of asymptomatic PAD in a population of 18,884 people between 45-74 years old and found that although the disease was diagnosed in 6.9% by an $ABI < 0.95$, only 22% of these people had symptoms of PAD. Table 2 shows similar data from the Rotterdam study highlighting that less than 50% of patients with PAD assessed by the ABI display symptoms of IC (Meijer et al 1998).

The BASLE study (LeFevre et al 1959) was amongst the first studies to measure the prevalence of asymptomatic disease using pulse waveforms detected on oscillography. Among men, the prevalence of occlusion confirmed by arteriography was 7.5% at those aged between 60-64 years old. Later, Criqui (Criqui et al 1985) used 3 different diagnostic tests (ABI, pulse wave analysis and pulse examination) and the WHO questionnaire in 613 men and women in California to define PAD. The prevalence of

PAD was 12% which was considerably higher than that estimated by the claudication questionnaire alone (2.2%).

The vast majority of epidemiological studies use the $ABI < 0.9$ alone to define asymptomatic PAD (DeBacker et al 1979; Fowkes et al 1991; Gofin et al 1987; Hughson et al 1978; Meijer et al 1998; Schroll et al 1981). Although there is not a valid cut-off point to define PAD, this is though to correlate well with the severity of disease across different populations (Fowkes 1991b). In the Edinburgh artery study 8% of those between 55-74 years had asymptomatic disease defined by the ABI and reactive hyperaemia test and a further 9% had $ABI < 0.9$ only (Fowkes et al 1991). Similarly, in the Golstrup study (Hughson et al 1978) 14% of a 60 years old population had $ABI < 0.9$. Moreover, from 2,174 individuals older than 40 years old of the National Health and Nutrition Examination Survey (NHANES), 4.3% had an abnormal ABI ($ABI < 0.9$) (Selvin et al 2004).

Recently, the PAD Awareness, Risk and Treatment (PARTNERS) study measured the ABI in 6,979 primary care patients aged 70 years or older or aged 50-69 years but with a history of smoking or diabetes (Hirsch et al 2001). The PARTNERS program focused on subjects at increased risk rather than the general population or a healthy group and found that PAD defined as $ABI < 0.9$ was present in 29% of the study population. This study provided further evidence that differences in prevalence of PAD between gender are very small, the prevalence of PAD in women was almost identical to than in men despite the fact that other CVD was almost twice as common in men than women.

The incidence of asymptomatic disease shows similar age and sex patterns as prevalence but has been studied less frequently. In the Basle study, for example, the 5 year cumulative incidence was 4% in men between 35 and 44 years old and 18% in men over 65 years of age (Widmer et al 1991). In 2,327 Dutch subjects, after 7.2 years, the overall incidence rate for asymptomatic PAD, assessed with ABI<0.9, was 9.9% per 1,000 person-years at risk (Hooi et al 2001). In this cohort, it was also noted that the incidence of asymptomatic PAD was higher than the incidence of symptomatic PAD, with women developing PAD more often than men.

Table 2 Prevalence estimates of peripheral arterial disease (PAD) by different diagnostic methods in the Rotterdam Study (Meijer et al 1998)

Age (years)	PAD by ABI<0.9 (%)	PAD by the WHO/IC questionnaire (%)
55-59	9.0	1.0
60-64	11.0	1.2
65-69	15.0	1.7
70-74	17.0	2.3
75-79	23.0	3.2
80-84	40.0	4.0
85-89	57.0	5.0

ABI: ankle brachial index, IC: intermittent claudication
Data modified from Meijer et al 1998

1.3.3 Critical limb ischaemia

There is little information on the incidence and prevalence of CLI. The Vascular Surgical Society of Great Britain and Ireland (The Vascular Surgery Society of Great Britain and Ireland 1995) estimated that there are approximately 20,000 people with CLI in the population with an annual incidence of 400 per million per year. Catalano (Catalano 1993) calculated the incidence of CLI using different approaches in an Italian population. After 7 years of follow up, of 200 people with IC and 180 controls, the incidence of CLI was 450 per million per year. Alternatively, looking at the hospitalizations for CLI for a sample of hospitals the incidence of CLI was 652 per million per year. Recently, the Oxford Vascular study followed up 19,106 subjects to record non-coronary event rates (Rothwell et al 2005). After 3 years, the rate for CLI was 0.34 per 1,000 population per year and 0.38 and 0.29 in males and females respectively.

On the assumption that all amputations are performed for CLI and that 25% of people with CLI require amputation the incidence of CLI can be estimated between 500-1,000 per million per year (Dormandy et al 2000). Another approach would be to look at the prevalence of IC and assume that 5% of claudicants will progress to CLI after 5 years. Then the incidence of CLI is approximately 300 per million per year and approximately one patient per year will develop CLI for every 100 with IC (Dormandy et al 2000).

Recently, Jensen et al used a questionnaire to estimate the prevalence of CLI in 20,291 Norwegian men and women aged 40-69 years (Jensen et al 2006). The authors defined CLI as ulcer in the toes, foot or ankle which failed to heal and/ or persistent pain in the forefoot while in supine position with relief of the pain when standing up. They found that the prevalence of CLI was 0.26% among men and 0.24% among women (Jensen et al 2006).

1.4 Risk factors for developing PAD

Specific risk factors have been associated with the development and progression of peripheral atherosclerosis. Epidemiological studies have shown that risk factors for PAD are almost identical to those associated with coronary heart disease (CHD). However, some such as smoking would appear to be particularly important in peripheral atherosclerosis. The importance of age and sex was already mentioned in the previous section which showed that prevalence and incidence of PAD increased with age and is slightly more common in males than females. Here, a brief review of epidemiologic data on well-established risk factors for the development of PAD is given.

1.4.1 Smoking

All epidemiological studies have confirmed that smoking is a strong risk factor for PAD (Criqui et al 1989;Fowkes et al 1992;Gofin et al 1987;Hughson et al 1978;Kannel et al 1985;Murabito et al 1997;Reunanen et al 1982;Schroll et al 1981). In the Framingham study, 78% of patients with IC were smokers (Freund et al 1993) whereas in the

Edinburgh Artery Study the risk of developing the disease was 3 fold higher in smokers than in non-smokers (Price et al 1999). Data from these two studies also showed that diagnosis of PAD was made almost a decade earlier in smokers than in non-smokers (Fowkes et al 1992;Kannel et al 1985). Another study reported a 16 fold increase in risk of PAD in current smokers and a 7 fold increase in ex-smokers compared to never smokers (Cole et al 1993). Moreover, the Reykjavik Study (Ingolfsson et al 1994) of males older than 18 years found an 8 to 10 fold increase in the risk of IC among smokers. A recent meta-analysis of 4 prospective and 13 cross-sectional studies reported that overall the prevalence of symptomatic PAD was increased 2.6 fold in current smokers and 2.3 fold in ex smokers (Willigendael et al 2004).

This importance of smoking in PAD has been shown to be stronger than in coronary artery disease (CAD). In the Framingham Study the increased risk in smokers compared to non smokers for PAD was double that for CAD (Kannel et al 1985). Similarly, in the Edinburgh Artery Study, smoking increased the risk of PAD (range of odds ratios, 1.8-5.6) more than heart disease (range of odds ratios, 1.1-1.6) (Fowkes et al 1992). Cigarette smoking was also shown to have an independent effect on PAD in multivariable analysis and a relatively greater effect compared to other risk factors (Fowkes 1991a;Hughson et al 1978). A clear dose-response relationship, with a strong increase in risk for PAD in heavy smokers, has also been reported. In the Reykjavik Study (Ingolfsson et al 1994) among 8,045 individuals followed for 18 years the rate of developing PAD compared to non-smokers was 2.6 for those smoking 1-14 cigarettes

per day, 7.7 for those smoking 15-24 cigarettes per day and 10.2 for those smoking 25 or more cigarettes per day.

1.4.2 Diabetes mellitus

Overall, epidemiological evidence confirms an approximately 2-4 fold increase in prevalence of PAD in people with diabetes compared to those without (Fowkes et al 1992;Gordon et al 1972;Kannel et al 1979;Kannel et al 1985;Murabito et al 1997). In studies using the ABI, the prevalence of PAD (defined as an ABI<0.90) in diabetic individuals ranges from 20% to 30% (Beks et al 1995;Hirsch et al 2001). Moreover, data from the Framingham and Rotterdam studies have shown increased rates of absent pedal pulses, femoral bruits, and decreased ABI in people with diabetes (Abbott et al 1990;Meijer et al 1998).

Diabetic PAD often affects distal limb vessels, such as the tibial and peroneal arteries, limiting the potential for collateral vessel development (Luscher et al 2003;Meijer et al 1998). As a result, patients with diabetes are more likely to develop severe symptomatic forms of the disease such as rest pain, gangrene or ulceration as well as IC (Jude et al 2001). In the Framingham study for both sexes, diabetes was associated with a 2 to 3 fold excess risk of IC compared with its absence (Brand et al 1989). Also, in 467 men and 1,444 women older than 80 years, the odds ratio for PAD in patients with diabetes was 6.1 in men and 3.6 in women compared to those without diabetes (Ness et al 2000).

The duration and severity of diabetes would appear to correlate with the incidence and extent of PAD (Jude et al 2001). A strongly positive association between the duration of diabetes and the risk of developing PAD especially among hypertensive men or current smokers was found (Al-Delaimy et al 2004). In addition, in the United Kingdom Prospective Diabetes Study (UKPDS) (Adler et al 2002) the frequency of PAD up to 18 years after the diagnosis of diabetes in 4,987 subjects was higher the longer the duration of diabetes.

1.4.3 Blood lipids

The effect of blood lipids on PAD is less clear and it has been suggested that the association between elevated cholesterol levels and PAD seems to be somewhat weaker than that for CHD (Pasternak et al 2004). In the Framingham study, people with IC had a higher mean cholesterol level and people with total cholesterol >270 mg/dl had twice the incidence of developing IC (Kannel et al 1970; Murabito et al 1997). Similarly, in the NHANES 2,174 subjects older than 40 years with a total cholesterol of 240 mg/dl or greater had a risk ratio of 1.88 for PAD defined by ABI<0.9 (Murabito et al 1997; Selvin et al 2004b). In a Danish cohort, total cholesterol was associated with decreased ABI 10 years later (Reunanen et al 1982). Also, a modest risk ratio of 1.10 for PAD (ABI<0.9) was found in the Cardiovascular Health Study for a 10mg/dl increase in total cholesterol (Newman et al 1993). Interestingly, these results were not replicated by other studies (Criqui et al 1989; Dagenais et al 1991; Hughson et al 1978; Zimmerman et al 1981).

Similar evidence was found for decreased HDL levels. In the Rotterdam study total cholesterol but not HDL cholesterol was associated with an ABI<0.9 (Meijer et al 1998). However, in the Edinburgh Artery Study both increased total and decreased HDL cholesterol were independently associated with IC and with ABI<0.9 (Fowkes et al 1992). Ness and colleagues, in a cohort of 467 men and 1,444 women, reported an odds ratio for PAD 0.95 in men and 0.97 in women for 1 mg/dl decrease in HDL levels (Ness et al 2000). The US physicians' study compared the LDL, HDL, triglycerides and ratio of total/ HDL cholesterol as predictors of PAD and found the latter to have the greatest independent effect (Ridker et al 2001). Evidence that treatment of hyperlipidemia reduces both the progression and incidence of PAD has also been found (Duffield et al 1983). Finally, an association between increased triglycerides and PAD has been reported by many but the strength of this association was reduced on multivariable analysis and thus effect of triglycerides on PAD remains unclear (Criqui et al 1989; Dormandy et al 1989; Fowkes et al 1992; Gofin et al 1987; Hughson et al 1978; Kannel et al 1985; Schroll et al 1981).

1.4.4 Hypertension

Epidemiologic studies have confirmed an association between increased blood pressure levels and PAD and they estimated that approximately 50-92% of claudicants have hypertension (Hirsch et al 2001; Makin et al 2001; Olin 2005). In the Cardiovascular Health Study 52% of patients with asymptomatic PAD ($ABI \leq 0.9$) had high blood pressure whereas hypertension was associated with the progression of PAD defined by

declining ABI after 6 years of follow up (Kennedy et al 2005;Newman et al 1993).

Follow up data from the Framingham Study demonstrated a 2 to 4 fold increased risk of developing IC in men and women with hypertension (Kannel et al 1970). In the Golstrup study significant correlations were found between initial elevated systolic and diastolic blood pressure and ABI after 10 years (Reunanen et al 1982). This was also suggested in the Edinburgh (Fowkes et al 1992) and the Basle study (Widmer et al 1991) but not by the Whitehall study (Smith et al 1990) which found no association between hypertension and PAD. Overall high blood pressure is likely to be a risk factor for PAD but may not be as important as others such as smoking.

1.4.5 Other risk factors

Obesity and low physical activity have shown strong associations with CHD (Wannamethee et al 2001;Yusuf et al 2005) but their relationship with PAD is not well established. Few studies have examined the relationship between obesity, mainly measured by the body mass index (BMI), and incident PAD and the overall evidence for an association was weak (Criqui et al 1989;Hooi et al 1998;Jensen et al 2005;Planas et al 2001). As in CHD, an alternative measure of body fat, such as waist circumference or waist to hip ratio, might show stronger associations with incident PAD; however, currently there are no data on this relationship. On the other hand, a physically active lifestyle has been associated with a reduced risk of developing PAD in two studies (Housley et al 1993;Planas et al 2001). However, physical activity may be particularly

difficult to study in PAD because symptomatic disease may result in reduced physical activity.

Some evidence for ethnic differences in the prevalence of PAD has also been reported. In the San Diego population study, black ethnicity was an independent risk factor for PAD at a magnitude similar to that of other established risk factors (Criqui et al 2005). Likewise, the NHANES found an increase in the risk of PAD among non-Hispanic black individuals (Selvin et al 2004); a result that has been shown by others (Newman et al 1993;Zheng et al 1997). The excess prevalence of PAD in blacks could not be explained by the excess of diabetes, hypertension, or other CVD risk factors or by any greater susceptibility to CVD risk factors.

A series of other potential risk factors have been associated with PAD along with coronary and cerebrovascular disease. These are often called non-traditional or non-conventional or novel risk factors because their importance in PAD and other manifestations of atherosclerosis awaits to be confirmed from epidemiological and clinical research. Currently there is burgeoning interest on the role of inflammation and haemostasis on atherosclerotic development and progression and the potential of inflammatory and haemostatic markers to serve as markers of atherosclerotic disease. The biology, epidemiology and pathophysiology of these markers in relation to atherosclerosis are described in the next chapter.

1.5 Progression of local disease in the legs

Atherosclerotic disease of the lower extremities can progress locally (in the legs) and may also lead to systemic manifestations of the disease such as CHD or stroke.

Interestingly, most people seem to experience other cardiovascular events and fewer have worsening PAD. The progression of the latter group is described here.

1.5.1 Intermittent claudication and asymptomatic disease

Intermittent claudication can progress in different ways including improvement or stabilization, or worsening of claudication which may then require surgical intervention and finally amputation (Dormandy et al 2000). Studies have shown that the majority of patients either improve or stabilize (Dormandy et al 2000). This can be clearly seen in a review of 10 trials of patients with IC where 75% of claudicants experienced stabilization of their symptoms over the next 5-18 years (Dormandy et al 1991). In the Framingham Study, only about 30% of patients with IC had persistent symptoms for a minimum of 4 years (Peabody et al 1974). Also, symptoms progressed in only about 15-30% of claudicants and, in total, 25% required surgery or experienced tissue loss and less than 4% of claudicants required a major amputation (Dormandy et al 1991;Imparato et al 1975;McDaniel et al 1989;Peabody et al 1974). Of the 116 cases of claudication in the Edinburgh Artery Study, 28.8% still had symptoms of IC, 8.2% had had a surgical intervention and 1.4 % had developed a leg ulcer after 5 years of follow up (Leng et al 1996b).

The progression of asymptomatic disease has been very little studied. The data available indicate a relatively benign course as far as the legs are concerned. In the Basle study, only 20% of people with asymptomatic PAD developed IC and 8% CLI over 10 years (Widmer et al 1991). In support of this study, a third of asymptomatic stenoses progressed over two years arteriographically (Walsh et al 1991). In the Edinburgh Artery Study, of the 345 asymptomatic PAD patients, 33 (9.6%) developed IC, and 3 (0.9%) underwent vascular surgery after 5 years of follow up. Progression of asymptomatic disease can also be measured via non-invasive modalities such as the ABI over time. However, the ABI is usually measured at one time point in epidemiological studies and information on its progression is limited.

1.5.2 Critical limb ischaemia

It is particularly difficult to describe the natural history of the critically ischaemic limb because most patients undergo some form of vascular intervention (Dormandy et al 2000). Limb loss in patients with CLI is more common than in claudicants and 10-40% of people with CLI need amputation (Dormandy et al 1989). The remaining patients often require supportive or medical treatment (Dormandy et al 2000). Results from two multicenter studies showed that, in one, 61% of patients with CLI had attempted revascularization and 7% a primary amputation after 1 year of follow up (Wolfe 1986). In the other study, 44.4% of patients with CLI had attempted revascularization and 9.4% amputation after 2 years of follow up (The ICAI group 1997). A more recent study

reported that after 6 months, 12% of 1,569 patients with CLI needed amputation and 17.9% had persistent CLI (Bertele et al 1999).

1.5.3 Risk factors for worsening PAD

Limited data are available concerning prognostic factors that play a role in the transition of asymptomatic PAD to symptomatic PAD. For the progression of symptomatic disease, the most important risk factors for the development of PAD, smoking and diabetes, have also been shown to have an effect on disease progression (Dormandy et al 2000). Patients with IC who continued to smoke have been shown to develop CLI more often than those patients with IC that quit smoking (Jonason et al 1987). Likewise, patients with IC who had a high load of lifetime smoking have required reconstructive vascular surgery 3 times more than those who smoked less (Cronenwett et al 1984). Finally, patients with IC who continued to smoke had higher amputation rates than those who quitted in two other studies (Cronenwett et al 1984;Faulkner et al 1983;Lassila et al 1988).

Overall, patients with diabetes have more aggressive progression of PAD. Patients with IC and diabetes have shown to have a 35% risk of sudden ischemia and 2% risk of major amputation compared to claudicants without diabetes who had 19% risk of sudden ischemia and 3% risk of major amputation (Cronenwett et al 1984;McDaniel et al 1989). In addition, amputation or gangrene was 10 times more frequent in subjects with diabetic PAD than in PAD patients without diabetes (Dormandy et al 1999).

The ABI has also been shown to be predictive of progressive PAD. In a prospective study of 1,969 claudicants, an ABI<0.5 was the most significant predictor of deterioration of PAD assessed by arterial surgery or intervention (Dormandy et al 1991). Also a low ABI was associated with 2.4 fold higher risk of local atherosclerotic progression after 6.5 years of follow up (Jelnes et al 1986). There is also some evidence that the nature and symptoms of claudication might be related to disease progression. In Bloor's classic follow up study, a patient with sudden onset of claudication was twice as likely to improve as a patient with gradual onset (Bloor 1961). Also, there is some evidence that claudication has a less severe course in women. Studies have reported that although the ratio between males and females is 2:1 in claudicants it increases between 3:1 and 13:1 in later stages of the disease (Fowkes 1991).

1.6 PAD and other cardiovascular diseases

1.6.1 PAD and co-existing CVD

The prognosis of patients with lower extremity PAD is characterized by an increased risk of cardiovascular ischaemic events at least partly due to concomitant CAD and cerebrovascular disease in these patients. This has been confirmed by epidemiological research measuring the co-prevalence of PAD with other manifestations of atherosclerosis. The reported co-prevalence is highly dependent on the sensitivity of diagnostic tools used to define atherosclerotic disease either PAD, CHD or stroke.

Overall, between 40-60% of claudicants suffer from co-existing CAD diagnosed by history of disease, clinical examination or electrocardiogram (ECG) and 26-50% of claudicants suffer from co-existing carotid disease diagnosed with duplex examination (Dormandy et al 2000). Aronow and colleagues studied the co-prevalence of PAD with other atherosclerotic manifestations in a long term care facility (Aronow et al 1994). This study showed that of 468 patients with PAD, 58% had CAD and 34% had suffered from ischaemic stroke (diagnosed with clinical history or ECG). Likewise, in a study of 1,802 men and women, 161 of 236 subjects (68%) with PAD had co-existent CAD and 100 of 236 subjects (42%) with PAD had co-existent cerebrovascular disease (Ness et al 2000). Similar findings were reported in the Whitehall Study which found the prevalence of CVD in patients with IC to be 54% (Smith et al 1990). In a Danish cohort, 44.3% of men and 31.2% of women with IC also suffered from CVD (Reunanen et al 1982). Finally, when angiography was used to define CVD the co-prevalence with IC was reported as high as 90% by two studies (Hertzer et al 1984; Valentine et al 1994).

The Edinburgh Artery Study and the PARTNERS showed that when PAD was defined by $ABI < 0.9$ then 71% and 56% of subjects had co-existing CVD, respectively (Fowkes et al 1991; Hirsch et al 2001). In the NHANES study, the prevalence of CHD was 24% and of stroke 11% among those with $ABI < 0.9$ whereas of those with normal ABI, 7% had CHD and 3% had stroke (Selvin et al 2004). Further evidence was found in the Cardiovascular Health Study, where ABI was inversely correlated with the number of patients suffering from myocardial infarction (MI), angina and congestive heart failure.

In addition, the ABI was reported to correlate well with other measures of arterial disease in other vascular beds. The Edinburgh Artery Study and the Rotterdam study have found, for example, that low ABI was associated with higher carotid intima media thickness (IMT) (Allan et al 1997;Bots et al 1994). In support of these results, the Multi-Ethnic Study of Atherosclerosis, showed that ABI was inversely associated with coronary artery calcium levels and carotid IMT (McDermott et al 2005b).

1.6.2 Intermittent claudication

Patients with IC have been shown to have an increased risk of angina, heart failure, fatal and non-fatal MI, fatal and non-fatal stroke and cardiovascular and all cause mortality as reported by large scale epidemiological studies (Ouriel 2001). Table 3 presents results from prospective studies on CVD and all cause mortality in patients with IC assessed by the WHO IC questionnaire and it shows that most of the total mortality could be attributed to cardiovascular death. For example, the Helsinki study (Reunanen et al 1982) found a 3 fold increase in the risk of cardiovascular mortality in male claudicants compared to males without claudication whereas in the Framingham study the annual mortality rate was 4% greater in claudicants (Kannel et al 1971).

Table 3 All cause and cardiovascular mortality in subjects with intermittent claudication (IC) assessed by the WHO questionnaire

Study	Population with IC (N)	Age (years)	Follow up (years)	Total mortality %	CVD mortality %
Helsinki Study (Reunanen et al 1982)	122 M 93 W	30–59	5	14.7 2.1	12.3 NR
Whitehall Study (Smith et al 1990)	147 MW§ 175 MW	40–64	17	38.1 40.0	30.6 24.0
Quebec Vascular Study (Dagenais et al 1991)	188 M	35–64	12	11.2	8.5
Edinburgh Artery Study (Leng et al 1996)	73 MW	55–74	5	19.2	13.4

NR: not reported

M: men, W: women

§ Probable IC || Possible IC

1.6.3 Asymptomatic disease

People with asymptomatic (subclinical) atherosclerosis measured by the ABI or other non-invasive modalities also have an increased risk of cardiovascular morbidity and mortality (Abbott et al 2000; Criqui et al 1992; Leng et al 1996; Newman et al 1993).

Table 4 summarizes results from prospective studies and shows that individuals with an ABI<0.9 are at increased risk of experiencing either coronary or cerebrovascular events. Moreover, a recent meta-analysis of 9 studies which followed up subjects prospectively showed that the specificity and sensitivity of a low ABI (ABI<0.9) to predict CHD were 16.5% and 92.5%, for incident stroke were 16.0% and 92.2% and for cardiovascular mortality were 41.0% and 87.9 %, respectively (Doobay et al 2005).

In the Edinburgh Artery Study, after 5 years of follow up, people with an ABI<0.9 at baseline had an increased risk of non-fatal MI (relative risk 1.38), stroke (1.98) and cardiovascular death (1.85) after adjustment for age, sex, coronary disease, and diabetes (Leng et al 1996). A later report from this study, with 12 years follow up, demonstrated that an ABI<0.9 had improved prediction of fatal MI over and above that of conventional risk factors (Lee et al 2004). In the Honolulu Heart Program, men older than 70 years with ABI<0.8 had a significantly higher risk of CHD (Abbott et al 2000). The Framingham study of 251 men and 423 women aged 80 and older showed that ABI<0.9 was associated with a 2 fold increase in the risk of stroke but not with the risk of CHD (Murabito et al 2003).

The Cardiovascular Health Study measured the ABI in adults older than 65 years of age and found that an ABI<0.9 was significantly associated with incident CVD and recurrent CVD (Newman et al 1999). Moreover, the Atherosclerosis Risk in Communities (ARIC) study examined the relation between ABI and incident stroke after 7 years and reported a more than 5 fold increase in the risk of stroke in people with ABI<0.8 (Tsai et al 2001). In another analysis of the ARIC study, ABI was weakly associated with recurrent CVD in the univariable analysis only (Wattanakit et al 2005). However, results from the ARIC study need to be interpreted with caution since only one leg was used for the calculation of the ABI and therefore the prevalence of disease is likely to be underestimated.

In addition, the Cardiovascular Health Study along with the Strong Heart Study has recently examined the prognostic significance of high ABI (O'Hare et al 2006;Resnick et al 2004). As previously described, in subjects with diabetes, the ABI is artificially high due to arterial calcification. With that perception, high ABI could be a measure of increased arterial stiffness and therefore predictive of adverse cardiovascular results. Most epidemiologic studies have excluded subjects with increased ABI above 1.4 or 1.5 from their analyses. The Cardiovascular Health Study and the Strong Heart Study did not make these exclusions and have recently found a U shaped relationship between ABI and the risk of mortality; however, these results need replication from other studies (O'Hare et al 2006;Resnick et al 2004).

Finally, a low ABI has been shown to be a strong predictor of mortality. An ABI <0.85 was associated with a more than 2 fold increase in all cause mortality, whereas a strong trend between increasing risk of death and decreasing ABI has also been reported (Leng et al 1996; Mckenna et al 1991).

Table 4 List of prospective studies that have evaluated the risk ratio (95% CI) of people with ABI<0.9 for the development of non-fatal coronary heart disease or stroke in the general population

Study (reference)	Population	Age (years)	Follow up (years)	Risk ratio of ABI ≤ 0.9 vs. >0.9 (95% CI)	
				Coronary heart disease	Stroke
Belgian study (Kornitzer et al 1995)	2,023 men	40–55	10	5.0 (p= 0.006)	NR
Edinburgh Artery Study (Leng et al 1996)	1,592 men and women	55-70	5	1.4 (0.9, 2.2)	2.0 (1.0, 3.8) [†]
Cardiovascular Health Study (Newman et al 1999)	5,888 men and women	≥65	6	2.0 (1.4, 3.0)	1.6 (1.1, 2.3)
Honolulu Heart Program (Abbott et al 2000)	2,863 men	71-93	3-6	3.3 (2.2, 4.9) 2.7 (1.6, 4.5)	2.4 (1.5, 4.0) [§] 2.0 (1.1, 3.5)
Atherosclerosis Risk in Communities Study (Tsai et al 2001)	14,839 men and women	45-64	7	NR	5.7 (2.8, 11.7) 1.9 (0.8, 4.8)
Framingham Offspring Study (Murabito et al 2003)	674 men and women	80 (mean)	4	1.2 (0.7, 2.1)	2.0 (1.1, 3.7) [‡]

NR: not reported

[†] adjusted for age, sex, coronary disease, and diabetes at baseline

[‡] adjusted for age, sex, and prevalent cardiovascular disease

[§]PAD defined by ABI<0.8. First risk ratio adjusted for age and second for cardiovascular risk factors

^{||} age and sex adjusted

1.6.4 Critical limb ischemia

In general, cardiovascular and all cause mortality among those with CLI is high. In addition, patients with CLI have an elevated risk of future MI, stroke and vascular death, 3 fold higher than patients with IC (Novo et al 2004). There are few studies which have followed up subjects with CLI. In one study, 20% of patients with CLI died within a year (Wolfe 1986). While in another, 40-70% died after 5 years of follow up (Dormandy et al 1999). Similar results were presented from a follow up study in Italy; of 574 patients diagnosed with CLI, 21.9% have died within one year (The ICAI group 1997). As expected, the overall incidence of vascular deaths was significantly higher than that of non-vascular deaths (34.5 vs. 8.5%).

1.7 Diagnosis of PAD

In general, clinical examination and measurement of PAD is a complex task due to the variable clinical and subclinical manifestations of the disease. Various diagnostic tests appropriate for the assessment of PAD in large scale community programs and epidemiological studies (non-invasive, easy to use, cheap, and reproducible) have been described. An overview of the most commonly used is presented here.

1.7.1 Questionnaires for intermittent claudication

Claudication is the symptom that is often associated with PAD. However, the diagnosis of claudication is not straightforward. IC must be differentiated from lower extremity

pain due to non-vascular causes. True claudication begins after a reproducible length of ambulation and disappears after the patient stops walking even if she or he remains standing.

For the diagnosis of IC in epidemiological research several questionnaires have been developed over the years. The WHO/ Rose claudication questionnaire is the most commonly used (Rose 1962). It defines claudication as the calf pain that occurs in one or both calves during exercise, which does not begin at rest, does not disappear while walking, is provoked by hurrying or walking uphill and makes the individual stop or decrease walking speed. However, the pain should cease within 10 minutes of rest. The severity of IC can be graded using this questionnaire to Grade I (symptoms occur while walking at an ordinary pace on the level) and Grade II (symptoms occur while walking uphill or in a hurry).

The Edinburgh Claudication Questionnaire (Leng et al 1992) is a modified version of the WHO questionnaire which does not include questions asking whether the individual experiences calf or calves pain while walking, whether the pain disappears while walking and what is done if pain is experienced while walking. This questionnaire includes a diagram of the lower extremities and defines claudication as pain in the calf even if pain is also identified in other areas. It also includes a definition of atypical claudication as pain in the thigh or buttock without calf pain.

Finally the San Diego Claudication Questionnaire (Criqui et al 1996) allowed for the individual to report pain specific to the right or left leg and to specify whether the pain is in the calves, thighs and/ or buttocks. This questionnaire defines the following categories: no pain, pain at rest, non-calf pain, non –Rose exercise calf pain and Rose claudication.

Although these questionnaires are useful especially in large epidemiological studies for the diagnosis of PAD, they seem to have high specificity but moderate sensitivity. For example, the Edinburgh Claudication questionnaire showed 85% sensitivity compared to 65% for the WHO questionnaire although the specificity was over 95% for both (Leng et al 1992). Most importantly, IC itself might be an insensitive marker of PAD according to recent evidence which shows a broader range of symptoms in patients with PAD (McDermott et al 1999; McDermott et al 2001).

1.7.2 The ankle brachial index

Travis Winsor was the first to notice in 1950 that the ankle pressure is usually decreased if the arteries in the lower limb are obstructed (Winsor 1950). Later studies showed that with increasing degrees of arterial narrowing there is a progressive fall in systolic blood pressure distal to the sites of involvement (Yao 1970). These observations have led to the development of methods that detect the pressure drop and associate it with atherosclerotic disease of the limbs.

Systolic blood pressure is expected to augment as it travels down the limb due to the muscular peripheral arteries as well as the summation of reflected pressure waves (Weitz et al 1996). Therefore, systolic pressures of the tibial arteries of each ankle should be greater or at least equal to those of the arm. The ratio of the ankle to the brachial systolic pressure, called the ABI or the ankle brachial pressure index (ABPI) or the ankle arm index (AAI), is the cornerstone of non-invasive assessment of the patients with symptomatic or asymptomatic PAD. The normal ABI should be greater or equal to 1. An ABI less than unity (or less than 0.9 in practice) at rest, indicates haemodynamically significant arterial obstruction in the legs (Halperin 2002).

The ABI has been validated against classic angiography to determine its sensitivity, specificity, and accuracy as a lower extremity PAD diagnostic tool but only in selected populations. For example, Fiegelson (Feigelson et al 1994) measured a sensitivity of 89% and a specificity of 99% compared with angiography, using only posterior tibial measurements with a threshold of 0.8. That study demonstrated that the ABI had a positive predictive value of 90%, a negative predictive value of 99%, and an overall accuracy of 98%. In accordance, Lijmer (Lijmer et al 1996) demonstrated that the sensitivity of the ABI < 0.91 to detect stenoses of 50% or more reduction in lumen diameter was 79% and the specificity was 96%. Nassoura et al. (Nassoura et al 1996) compared the ABI with angiography after vascular occlusive injury due to trauma and demonstrated that the ABI had a sensitivity of 72%, a specificity of 100%, a positive predictive value of 100% and a negative predictive value of 96%. However, all these

reported specificity and sensitivity for cut-off points of ABI have been calculated using selected hospital populations and is unknown how ABI would correlate with angiographically measured peripheral atheroma in the general population (Fowkes 1991b). Thus, there is not a universally valid cut-off point below which people may be considered to have defined severity of atherosclerotic disease. Alternatively, abnormal ABI values represent a continuous variable less than a dichotomous value (above or below 0.90). Therefore, in patients with IC, the ABI is expected to decrease between 0.6-0.9, while in those with rest pain and tissue loss usually the ABI is <0.4 and <0.25, respectively (Ouriel 2001).

A limitation of the ABI is that it may not be accurate in individuals with non-compressible pedal arteries due to diabetes or very old age. Variations of the ABI have been designed for those individuals and they are described in the next section.

Methodology of ABI

The ankle systolic pressure can be measured by a cuff inflated proximally to the ankle and the detection of blood flow by means of a Doppler probe. A stethoscope is insufficiently sensitive in detecting pulses at the ankle. Either the posterior tibial and/ or the dorsalis pedis artery's pulse are detected or the peroneal artery if no audible signal is forthcoming from these two vessels.

In epidemiological surveys the calculation of the ABI varies while several methods for its measurement have been described. In most cases, the ankle systolic pressure is measured in both legs and similarly the brachial systolic pressure is measured in both arms which lead to at least 4 ratios of ABI. However, only one ratio is used to diagnose PAD. In some reports, the highest arterial pressure of either the dorsalis pedis or the posterior tibial artery in each leg is used to calculate the ABI (Mckenna et al 1991;McLafferty et al 1997), while in others the lowest arterial pressure in each leg is used (McDermott et al 1998). Alternatively, the dorsalis pedis and posterior tibial arterial pressures within the leg are averaged (Vogt et al 1993), or the posterior tibial arterial pressures in the right and left legs are averaged (Newman et al 1993;Vogt et al 1993). In other studies including the Framingham study, the Rotterdam study and the Edinburgh Artery Study, the ankle systolic pressure was measured in the posterior tibial artery and if that wasn't found in the dorsalis pedis in each leg (Fowkes et al 1991;Meijer et al 1998;Murabito et al 2003;Vogt et al 1993). Also, the ARIC study randomly selected one leg to measure the ankle systolic pressure. One brachial pressure is also measured in some studies; usually the right (Newman et al 1993;Zheng et al 1997).

After measuring the ankle and the brachial systolic pressures, in most studies, the ABI is calculated for each leg by dividing the left ankle to left arm pressures and right ankle to right arm pressures. The lowest of these ratios is used as it denotes disease severity. If only the right arm was used then the two ratios have the right brachial index as the denominator (Zheng et al 1997).

Currently there are no recommendations for the most appropriate measurement of the ABI. Some studies have used only the right brachial systolic pressure for the calculation of the ABI. However, patients at risk for arterial disease in the lower extremities often have concomitant occlusive arterial disease in the upper extremities and it is possible that the right and left brachial pressures may be discrepant (Federman et al 2006). It has been estimated that the pressure difference between the right and left brachial arteries is at least 20 mmHg is present in 3.5% of the normal healthy population and in over 20% of patients with PAD (Caruana et al 2005). If for example the left brachial pressure is higher than the right for a sizable proportion of subjects, the calculation of the ABI using only the right systolic pressure might be biased so its is probably vital to measure both brachial pressures.

In addition, recent studies comparing different methods of calculating the ABI agreed that lower ABI taken by averaging the dorsalis pedis and posterior tibial arterial pressures in each leg was most predictive of walking endurance and walking velocity in PAD and had lower intra- and inter-observer variability (Aboyans et al 2003). However, in epidemiologic research where thousands of people are undergoing a specific test, taking measurement from both arteries of the ankle might be particularly difficult and time consuming. In this case, the measurement of posterior tibial and if not present the dorsalis pedis should be a good marker of the underlying atherosclerosis.

Finally, the method used to measure the systolic pressure may differ across different studies. The traditionally used mercury sphygmomanometers are less used due to safety reasons and automatic devices are taking their place (Caruana et al 2005). Few studies have evaluated the use of these automatic devices but the results show that is still probably inadequate. Lee (Lee et al 1996) and Mundt (Mundt et al 1992) concluded that results taken with automatic device are the same with these taken with a cuff and a Doppler probe. On the contrary, in another study there was no correlation between values obtained with a Doppler technique and those obtained by an automatic device and the latter overestimated the ankle pressure (Jonsson et al 2001).

Variability of ABI

Few studies have examined the variability of ABI and concluded that it is such that ABI is a suitable measurement for epidemiological studies (Caruana et al 2005). Overall, the ABI is considered to have a reproducibility of approximately 0.10 (Hirsch et al 2006). Early research by Carter (Carter 1968) showed that 95% of repeat measures of ankle blood pressures were within 9% of the average; a result which is similar to that obtained when measuring brachial systolic pressure. The intra-observer variability of the test was estimated by Fowkes (Fowkes et al 1988) as 7.2% in diseased subjects and 10.4% in normal subjects. Kaiser (Kaiser et al 1999) reported 11.8% variability when measurement was taken by a single observer. The latter study also pointed out that trained observers might have significantly decreased intra-observer variability as low as $\approx 7\%$. Yao (Yao 1970) found the measurement of ABI as 0.08 assessed in 179 patients

on different days using 4 technicians. When 69 patients were assessed on 6 different days using the same technician, the measurement variance decreased to 0.05. These results suggest that most of the variance is attributed to the measurement method and less due to inter-observer differences.

1.7.3 Other diagnostic tests

In addition to the ABI, a number of other diagnostic tests have been developed for the diagnosis of PAD. For example, the sensitivity of the ABI can be increased with a stress test. A similar test which might be more appropriate for epidemiological surveys is the reactive hyperaemia test. This is conducted at rest and uses simpler equipment. The blood flow is occluded at the upper thigh or above the knee and the systolic blood pressure is measured after releasing the obstruction (Johnson 1975). Moreover, along with the ABI, multiple pressure measurements can be calculated along the lower extremity which may help localize the site of arterial obstruction. Finally, in elderly subjects and in subjects with diabetes, the arteries are often calcified and thus incompressible and the ABI can be artificially elevated. In these situations, toe pressures can also be informative.

The anatomical level of the disease can also be identified by the palpation of pulses in the femoral, popliteal and ankle regions. However, pulse palpation is of limited use in epidemiologic research due to its low sensitivity, high variability and its discrete

(qualitative not quantitative) measure of perfusion (Brearley et al 1992;Myers et al 1987).

Another diagnostic test is flow velocity determination. This diagnostic test does not provide visualization of the arterial anatomy but can be useful in assessing PAD anatomy, severity and progression (Hirsch et al 2006). It should be noted that it has greater specificity for superficial femoral arteries than for aortoiliac occlusive disease (Hirsch et al 2006). Other disadvantages include limited accuracy in tortuous, overlapping or calcified arteries and high dependence on the operator (Strandness et al 1967). Alternatively, duplex ultrasound which combines B-mode and Doppler ultrasound measurements and can provide information concerning artery wall thickness, degree of flow turbulence, changes in blood flow and vessel morphologic characteristics (Kohler et al 1990;Strandness et al 1967).

Finally, angiography or arteriography remains the gold standard for the diagnosis of PAD with which the specificity and sensitivity of all other tests are compared. This is an invasive test and it is only used in those people that surgical intervention is planned because angiography is associated with both local and systemic complications, morbidity and mortality. Alternatively, magnetic resonance angiography and computerized tomography arteriography can be used which contrary to traditional angiography, are not associated with increased risk of stroke or of arterial injury. However, their correlation with angiography needs to be established (Halperin 2002).

1.8 Summary

PAD is a manifestation of atherosclerotic disease in the arteries to the legs. The clinical presentation of PAD includes a spectrum which spans individuals with asymptomatic disease, those who experience IC and those with more severe symptoms of CLI.

Epidemiologic research has confirmed that PAD is a common condition which affects a large proportion of the adult population worldwide. The estimated prevalence of claudication ranges from 0.4 to 14.4%. The prevalence of asymptomatic disease diagnosed with non-invasive tests is much higher and ranges between 0.9% and 22% with the ratio of symptomatic to asymptomatic ranging between 1:0.9 and 1:6.0. Risk factors for atherosclerosis such as age, cigarette smoking, diabetes, dyslipidemia, and hypertension increase the likelihood of developing PAD. Smoking and diabetes have also been associated with the progression of symptomatic disease. Risk factors for the progression of asymptomatic disease have been very little studied.

The majority of patients with IC experience stabilization of their symptoms in the next 5 years and only 10-15% ever develop CLI. The progression in the legs of those with asymptomatic disease is very little studied. However, both claudicants and those with asymptomatic disease are at increased risk of systemic cardiovascular events.

The diagnosis of PAD in epidemiologic studies is usually performed by non-invasive diagnostic tests. The ABI is the most commonly used non-invasive test and can be considered as an accurate and reliable marker of subclinical peripheral and generalized

atherosclerosis in populations. Indeed, a decreased ABI (usually below 0.9) has been associated with increased risk of MI, stroke and other manifestations of atherosclerosis and with other non-invasive test of subclinical disease in other vascular beds.

Thus PAD is a common condition especially if asymptomatic individuals are accounted for. However, the natural history of asymptomatic disease is not well established.

Specific risk factors for the progression of asymptomatic disease need to be established to help the identification of seemingly healthy individuals who are at increased risk of experiencing a cardiovascular event and to provide insights into the early stages of disease development.

Chapter Two

2 Inflammation and haemostasis

2.1 Introduction

The previous chapter focused on the epidemiology of PAD- an atherosclerotic disease of the lower extremities. Several mechanisms have been proposed for the initiation and accumulation of atherosclerosis in the peripheral and other vascular beds. Historically, the aging hypothesis proposed that atherosclerosis is a process of the aging of the arteries. This was replaced by the lipid hypothesis which stated that the accumulation of lipids due to diet and genetic factors result in atherosclerotic lesions in the arteries.

Latterly, with advances of molecular and vascular biology, the role of haemostasis and inflammation in atherosclerotic development and progression was recognized.

Fundamental research since then has shown that these two universal responses to injury seem to interact and may play a key role in the initiation and propagation of atherosclerosis.

This chapter presents a brief overview of the inflammatory and haemostatic mechanisms and their interactions. It also describes the hypothesized role of these mechanisms in the atherosclerotic process and focuses on specific biological factors that may serve as markers of the ongoing inflammation and haemostatic process and therefore of the

atherosclerotic process. The biological structure and function of each marker and a summary of their measurement methods is described here.

Many studies have examined the role of these biomarkers in CVD. A literature review on epidemiological studies of these markers and different CVD endpoints is also included in this chapter. The literature review included only studies in the English language and with prospective design which were sought by MEDLINE searches. Computer searches used combinations of key words relating to the blood factors (e.g., fibrinogen, C-reactive protein, CRP, interleukin-6, IL-6, D-dimer, acute phase reactants) and to the disease of interest (e.g., coronary heart disease, myocardial infarction, atherosclerosis, peripheral arterial disease). References cited in the articles identified through the literature review were also examined. Some examples of cross-sectional or case control studies were also mentioned. Also, since the Edinburgh Artery Study is a population based cohort study, the literature review included studies on healthy or apparently healthy individuals. However, studies on selected population groups (people with pre-existing CVD) were summarized. Because a meta-analysis has already been published for the majority of the markers mentioned here, a systematic review was not performed. Moreover, because this thesis is on epidemiology, animal studies and basic biological studies are not reported. Finally, studies on genetic epidemiology of these biomarkers were not an objective of this thesis and therefore were not included in the literature review.

2.2 Overview of haemostasis

Haemostasis is the physiological response to blood loss following vascular injury and thus an important defence mechanism against bleeding. The process produces a fibrin platelet clot which effectively seals the damaged blood vessel. Generation of the clot and subsequent clot lysis involves complex interactions between vascular endothelium, blood platelets, coagulation factors and components of the fibrinolytic system which are finely regulated to localise the clot to the area of damage.

2.2.1 Coagulation

The main role of the coagulation system is to produce thrombin from its precursor prothrombin. The precise and balanced generation of thrombin at sites of vascular injury is the result of an ordered series of reactions collectively referred to as blood coagulation (Mann 1999). Thrombin is a protein with many biologically important functions such as the activation of platelets, conversion of fibrinogen to a fibrin network and feedback amplification of coagulation (Dahlback 2005).

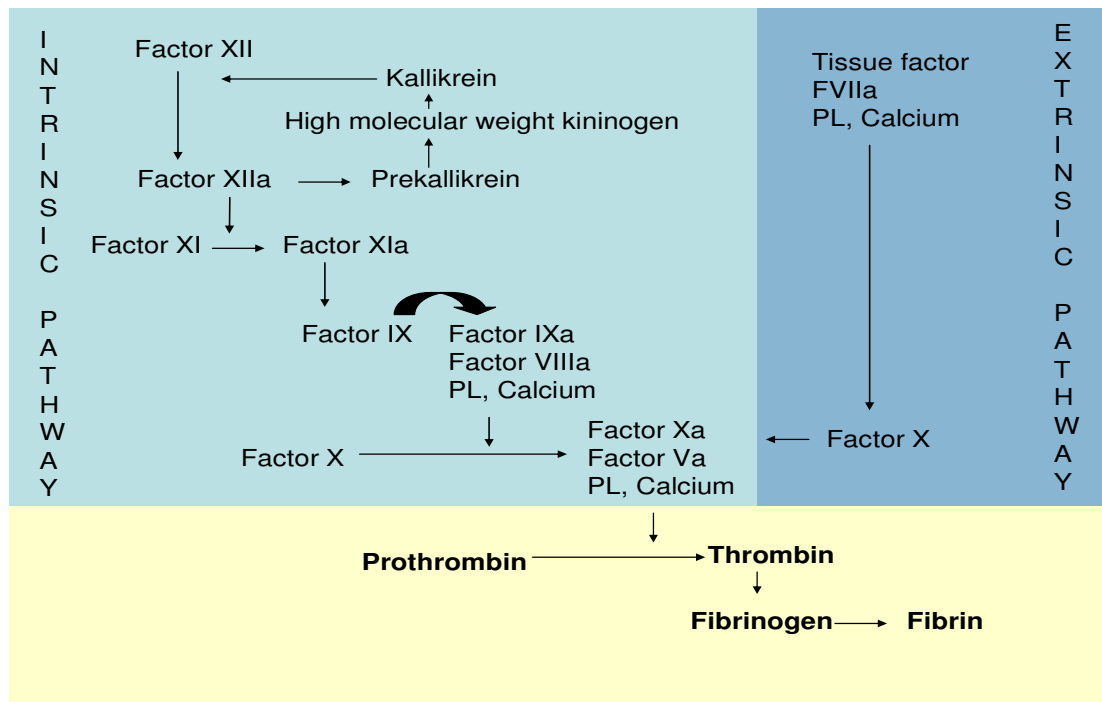
More than 35 years ago, Mc Farlane (Macfarlane 1964) and Davie and Ratnoff (Davie & Ratnoff 1964) proposed a cascade/ waterfall model of coagulation. This was the first step towards the understanding of individual coagulation proteins and their interactions (Becker 2005). According to the cascade model, two distinct systems of fibrin formation exist, the extrinsic (tissue factor) and the intrinsic (contact) pathway. These pathways are outlined in a Y-shaped scheme, with the distinct “intrinsic” and “extrinsic” pathways

initiated by factor XII (FXII) and FVIIa/ tissue factor, respectively (Figure 1). The pathways eventually converge on a “common” pathway where factor Xa converts prothrombin to thrombin which then acts on fibrinogen. The coagulation complexes are generally noted to require phospholipid and calcium for their activity.

Research over the past decade showed that these two pathways cannot operate independently as was initially proposed in the cascade model (Hoffman et al 2001). In fact, these two systems seem to interact extensively so that coagulation in vivo results from a complex and multidimensional system (Becker 2005). Hoffman (Hoffman et al 2001) proposed a cell based approach to coagulation. He suggested that rather than conceiving coagulation as only a "cascade" of proteolytic reactions, the coagulation reactions occur as overlapping steps: initiation, amplification, and propagation. Under this model, the extrinsic and intrinsic pathways play distinct and complementary roles (Hoffman et al 2001).

A number of mechanisms prevent initiation of coagulation under normal circumstances. Among them, the three dominant anti-coagulant mechanisms include the heparin – antithrombotic system, protein C-anticoagulant pathway and tissue factor pathway inhibitor (Esmon 2004).

Figure 1 The coagulation cascade



2.2.2 Fibrinolysis

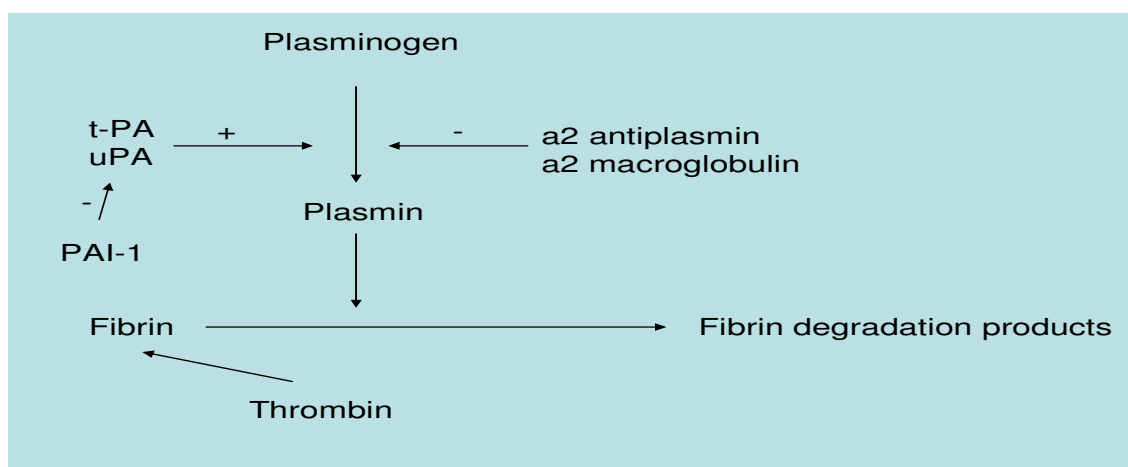
Once haemostasis is restored and the tissue is repaired the clot must be removed from the injured tissue. This is achieved by the fibrinolytic pathway which is illustrated on Figure 2. Plasmin is the major fibrinolytic protease. Plasminogen, a circulating plasma zymogen, can be converted to plasmin by both tissue plasminogen activator (t-PA) as well as by urokinase (u-PA). The t-PA pathway is primarily involved in fibrin haemostasis whereas the u-PA is primarily involved in tissue remodelling (Collen 1999).

Once it is formed, plasmin degrades fibrin and can activate metalloproteinases which in turn degrade the extracellular matrix (Collen et al 1991). Fibrin, the major plasmin

substrate, regulates its own degradation by binding both plasminogen and t-PA on its surface, promoting plasmin generation (Cesarman-Maus et al 2005).

Inhibition of the fibrinolytic system can occur by several mechanisms. Three of these include the plasminogen activator inhibitor-1 and plasminogen activator inhibitor-2 (PAI-1 and PAI-2) which inhibit plasminogen activators, alpha 2-antiplasmin and alpha 2-macroglobulin which inactivate plasmin and the tissue inhibitors of metalloproteinases (Collen 1999).

Figure 2 The fibrinolytic system



2.3 Overview of inflammation

Inflammation is the body's first line of defence against injury or invasion. The purpose of inflammation is to limit damage to the body after injury such as abrasions and lacerations or invasion by foreign organisms, such as bacteria or viruses. The principal signs of inflammation—redness, heat, pain, and swelling—are easily perceptible. On a microscopic level, the inflammatory response involves numerous different chemicals, each performing a specific action.

2.3.1 Acute inflammation

The inflammatory process can be divided into three major and interrelated components: vascular dilation, endothelial activation and neutrophil activation (Stevens et al 2002a).

The earliest event of an inflammatory response is vasodilation. Relaxation of the vascular smooth muscle leads to increased permeability and blood flow (hyperaemia) that causes redness (erythema) and the entry of fluid into the tissues (oedema).

Due to increased permeability, plasma proteins pass into the tissues that cause the expression of adhesion molecules. Adhesion molecules mediate leukocyte adherence. The leukocytes, in particular neutrophils, attach themselves to the endothelial cells within the blood vessels and then migrate into the surrounding tissue. The complement cascade is then initiated forming several key plasma mediators of inflammation. If the

vessel wall is damaged fibrinogen is cleaved to form fibrin at the site of injury, platelets aggregate and become activated, and the red cells aggregate to help stop bleeding and lead eventually to clot formation.

Possible outcomes of acute inflammation include resolution (meaning that the normal tissue architecture is restored) or scarring (fibrosis) when tissue is unable to regenerate and excessive fibrin deposition occurs at the site of injury (Stevens et al 2002a). Also, abscess formation can occur with some infections or progression to chronic inflammation may follow.

2.3.2 Chronic inflammation

Chronic inflammation is characterized by inflammatory processes which continue for a relatively long duration. Essentially, chronic inflammation is caused by one of two means, either by a persistent inflammatory stimulus or ineffective inflammatory mechanisms.

Chronic inflammation involves monocytes, macrophages, lymphocytes and plasma cells (Stevens et al 2002b). The sequence of chronic inflammation centres around the activity of macrophages derived from blood monocytes. Once at the site of inflammation, the

macrophage is incapable of completing its role. As a consequence, more monocytes are recruited from the blood, more macrophages proliferate locally, and both groups are effectively stranded at the site. Increased macrophage activity and local destruction liberates a number of cytokines including interleukins and growth factors. These attract a range of supplementary cells including lymphocytes, fibroblasts, plasma cells, eosinophils and mast cells. The interaction between various cells is essential: lymphocytes and macrophages act in a complementary manner, each inducing the other's proliferation and differentiation via mediators such as interferon and interleukins. Growth factors cause random matrix-deposition, angiogenesis and epithelialization.

2.3.3 The acute phase response

Inflammation, either chronic or acute, is accompanied by systemic and metabolic changes collective known as the acute phase response (Kushner 1982, Baumann 1994). The purpose of this response is to restore haemostasis and to remove the cause of disturbance.

The acute phase response is characterized by the activation of the complement and the blood coagulation system, hormonal changes and changes in the lipid metabolism and induction of acute phase proteins (Whicher et al 1992). The acute phase proteins are defined as those 30 or so plasma proteins which increase in concentration by 25% or more in the first 7 days following tissue damage. They are widely measured to indicate

the presence of inflammation and its extent or severity (Kushner 1982). Most acute phase proteins are produced in the liver but some are produced by other cell types, including monocytes, endothelial cells, fibroblasts and adipocytes (Gabay et al 1999). The most common acute phase proteins are listed in Table 5. Major acute phase proteins can increase concentration to 1,000 fold over normal levels. This group includes serum amyloid A (SAA), C-reactive protein (CRP) and serum amyloid P component (SAP). So-called negative acute phase proteins are decreased in plasma concentration during the acute phase response.

The function of most acute phase proteins has not been totally elucidated. They seem to have a wide range of activities that contribute to host defence: they can directly neutralize inflammatory mediators, limit the extent of local tissue damage, as well as participate in tissue repair and regeneration (Baumann et al 1994). Some proteins also have anti-inflammatory properties, whereas, coagulation components, such as fibrinogen, play an essential role in promoting wound healing (Gabay et al 1999). The interleukin-6 (IL-6) family of cytokines are the major inducers of acute phase protein synthesis in experimental studies, with IL-1 and tumor necrosis factor (TNF) playing a minor role.

Table 5 Positive and negative acute phase proteins (Gabay et al 1999)

Proteins whose levels increase	
Complement proteins	C3, C4, C9, factor B, C1 inhibitor, C4b binding protein, mannose binding lectin
Proteins of the haemostatic system	fibrinogen, plasminogen, tissue plasminogen activator, urokinase, protein S, vitronectin, plasminogen-activator inhibitor-1
Proteins of the inflammation system	phospholipase A2, liposacharide
Antiproteases	a1 protease inhibitor, a1 antichymotripsin
Transport proteins	cerulopasmin, haptoglobin, hemopexin
Others	C-reactive protein, serum amyloid A, fibronectin, ferritin, angiotensinogen
Proteins whose levels decrease	
	albumin, transferrin, factor XII, transthyretin, a- fetoprotein

2.4 Inflammation and haemostasis in atherosclerosis

There is compelling evidence from both clinical and basic biological studies that the aforementioned physiological mechanisms originally involved in defence against the hostile world, play an important role in the initiation and progression of atherosclerotic disease. However, the exact underlying cellular and molecular pathways that predispose to atherosclerosis are not fully known yet and several models have been proposed to explain the initiation and progression of this complex disease. Here, the current evidence on the pathophysiology of atherosclerosis is summarized, with emphasis on the

inflammatory or haemostatic mechanisms that have been proposed as participates in several stages of disease development and progression.

2.4.1 Formation of atherosclerotic plaque

Inflammation and haemostasis are believed to participate in atherosclerosis from its inception. Endothelial dysfunction is one of the earliest events that contribute to atherosclerotic development. Normally, leukocytes are unable to attach to the arterial wall. However, the expression of adhesion molecules by the endothelial cells permits the attachment of leukocytes to the arterial wall. This first step includes the rolling of leucocytes along the endothelial surface and is mediated by selectins. Fibrinogen also binds to adhesion molecules and causes the release of vasoactive mediators and modulates the endothelial cell's permeability (Koenig 2003).

Once adhered to the arterial endothelium, leukocytes- mainly monocytes- transmigrate through the endothelial lining to the intima. This process is induced by chemokines. Once the monocytes reach the intima they mature into macrophages and participate in and perpetuate a local inflammation response (Libby 2002). The macrophages express scavenger receptors, engulf modified lipoproteins and become foam cells. Additional injurious stimuli continue the attraction of macrophages, mast cells and activated T cells into the developing lesion (Pearson et al 2003). Further release of cytokines, chemokines and growth factors induce further damage and eventually lead to focal necrosis. Inflammatory cytokines initiate coagulation through the induction of tissue factor

expression, primarily on monocytes (Edgington et al 1991). Also, primary cytokines induce the expression of IL-6 which further induces the expression of hepatic genes that encode acute phase reactants including CRP and fibrinogen (Tousoulis et al 2003). New cycles of accumulation of mononuclear cells, migration and proliferation of smooth muscle cells and formation of fibrous tissue lead to the forming of a fibrous cap which overlies a core of lipid and necrotic tissue (Ross 1999).

2.4.2 Plaque rupture

During the atherosclerotic development the disease is often asymptomatic. However, there is an increased chance of plaque rupture and activation of the coagulation system. Atherosclerotic plaques that are at increased risk of rupture are called vulnerable or high-risk plaques (Fuster et al 2005). In general, if the thrombi are small they contribute to the growth of the atherosclerotic plaque; however, if they are large they may lead to acute syndromes. Notably, in some patients with acute syndromes, there is no plaque rupture but only superficial erosions.

The fibrous cap, which is located on top of the atherosclerotic plaque, protects the blood from contacting the lipid core of the plaque. Therefore, disruption of the atherosclerotic plaque is usually a result of fibrous cap rupture. The fibrous cap is stabilized through collagen. However, activated macrophages secrete connective tissue enzymes, tissue factor and metalloproteinases which may break down collagen, weaken the fibrous cap and contribute significantly to its rupture (Libby 1996). Rupture of the fibrous cap

exposes thrombogenic and procoagulant material initiating the coagulation cascade (Virmanyi et al 2000). Platelet activation and aggregation, thrombin generation and the development of a large potentially occlusive thrombus follow (Tracy 2003).

2.4.3 Markers of inflammation and haemostasis

As described in the previous sections, numerous activated molecules are involved in inflammatory and haemostatic responses. Levels of such molecules may serve as attractive measurements of the ongoing inflammation or haemostatic processes and therefore potentially of the atherosclerotic process. Indeed, a series of inflammatory and haemostatic markers have been thoroughly examined in epidemiological research over the past 20 years to test this hypothesis. The observed associations are of particular interest since they may provide further insights into the pathophysiological mechanisms of atherosclerosis and also yield predictive and prognostic information of clinical utility.

This thesis examines the epidemiology of inflammatory and haemostatic molecules in the atherosclerotic process focusing on peripheral atherosclerosis. Five markers of inflammation and seven markers of haemostasis were included in the analysis. However, as described previously, the boundaries between inflammation and haemostasis often overlap and some markers might reflect both mechanisms. The following section briefly describes the function of each marker under investigation and summarizes the epidemiological evidence between each marker and atherosclerotic disease.

2.5 C-reactive protein

2.5.1 Structure and function

CRP was first detected in 1930 in the sera of patients acutely infected with pneumococcal pneumonia and named by its reactivity with polysaccharide C of *Streptococcus pneumonia* (Tillett et al 1930). It is a calcium-binding pentameric protein consisting of five identical non-glycosylated polypeptide 23-kd subunits. It is present in trace amount in humans and has been highly conserved over hundreds of millions of years.

CRP is an acute phase reactant. It is produced by hepatocytes predominately within hours of an injury or the presence of inflammation under the control of IL-6 but also of IL-1 and TNF (Li et al 2004). Its concentration in acute inflammation is raised by 1,000 fold or more. CRP may also be produced away from the liver by vascular sources including cells residing in atherosclerotic plaques (Lind 2003). Its levels remain elevated throughout the acute phase response until normal tissue function and structure are restored. Normal CRP levels are generally defined <10 mg/L and healthy individuals generally have <1mg/L (Armani et al 2005).

Its role its not fully understood but two functional properties of CRP including the ability to activate complement through the classic complement pathway, and the ability to modulate the function of phagocytic cells have been demonstrated (Li et al 2004). It is also known to bind in a wide variety of substances such as microbial polysaccharides,

damaged cell membranes (Volanakis et al 1979), LDL in vitro (Pepys et al 1985) and apoptotic cells (Gershov et al 2000). Moreover, it has been shown that CRP helps the induction of inflammatory cytokines and tissue factor in monocytes (Cermak et al 1993) and that it activates endothelial cells to produce VCAM-1, ICAM-1 and E-selectin (Pasceri et al 2000).

2.5.2 Measurement

Low levels of CRP are easily and inexpensively measured. Standardized high-sensitivity assays are commercially available for this purpose and provide similar results in stored, fresh or frozen plasma (Koenig 2005). The high sensitivity CRP assay detects levels of CRP from 0-2 mg/L and their coefficient of variation is generally <10% (Pearson et al 2003). Several high sensitivity CRP assays have been described including immunonephelometric, immunoturbidimetric and immunoluminometric methodology. Fasting samples are not required. Also, samples have been shown to be stable at room temperature and there are no variations with time of the day (Ockene 2001; Wilson 2003). Moreover, the variability and classification accuracy of CRP is similar to that of total cholesterol (Ockene et al 2001).

CRP levels are known to be higher among patients with traditional cardiovascular risk factors. Obesity, increased blood pressure, dyslipidemia, diabetes and the metabolic syndrome are associated with elevated CRP (Pearson et al 1997). Similarly, moderate alcohol use (Sierksma et al 2002), increased physical activity (Kasapis et al 2005), high

fiber diet (Ma et al 2006) and medications, such as statins, are associated with decreased CRP levels (Strandberg et al 1999). CRP is also elevated in the first trimester of pregnancy and in women taking oral contraceptives or hormone replacement therapy (Gol et al 2006; Ridker et al 1999).

2.5.3 C-reactive protein and CVD

CRP is the most thoroughly investigated inflammatory marker. One of the earliest studies on CRP, in 1982, showed that peak CRP concentrations correlated with myocardial ischemia and necrosis (de Beer FC et al 1982). From that time, levels of CRP have been found to predict future risk among selected populations including patients with unstable angina, acute coronary syndromes, stable coronary disease, metabolic syndrome and among patients undergoing revascularization procedures (Blake et al 2002; de Ferranti et al 2002; Haverkate et al 1997; Liuzzo et al 1994; Ridker et al 2003; Ridker et al 2001; Rifai et al 2001).

There is now a wealth of evidence from large-scale prospective studies of apparently healthy individuals that baseline levels of CRP are associated with future cardiovascular events (Koenig 2005). Up to date, at least 33 prospective epidemiological studies have tested the association between CRP and incident CVD in healthy populations (Table 6). As Table 6 shows, the vast majority of these studies have focused on coronary events either MI or CHD, and much fewer on stroke events.

Interestingly, the earlier studies have found significant associations between CRP and incident CVD, CHD or MI which were independent of traditional cardiovascular risk factors (Table 6). However, several later studies failed to replicate these results. For example, data from the ARIC (Folsom et al 2002), the health ABC (Cesari et al 2003) and the British Women's Heart and Health study (Lawlor et al 2005) did not show significant associations between CRP and future CHD in the multivariable analyses. In support, meta-analysis of most published studies up to year 2000 (Danesh et al 2000) showed a relative risk (95% CI) of 2.0 (1.6, 2.5) in subjects with single CRP baseline measurement in the upper tertile compared with the lower tertile. However, an updated meta-analysis (Danesh et al 2004) showed a much smaller relative risk (95% CI) of about 1.5 (1.37, 1.62). In fact, despite the fact the CRP is often cited as having consistent results and strong and independent associations with incident CVD in many epidemiological studies, careful investigation of Table 6 reveals that the effect of CRP might be modest and that, in at least 13 studies, did not retain statistical significance in the multivariable model.

Nevertheless, CRP has been found to confer additional prognostic value to that of other biomarkers such as lipid levels, lipoprotein (a) and other inflammatory markers in one study (Ridker et al 2000a). In addition, CRP was also found superior even to LDL cholesterol levels (Ridker et al 2002) and proved to enhance global coronary risk as assessed by the Framingham Risk Score (Koenig et al 2004; Wilson et al 2003). However, in several other studies, CRP failed to improve significantly the c-statistics of

the Framingham risk function alone (Danesh et al 2004;Rutter et al 2004;van der Meer et al 2003).

Two cross-sectional studies have indicated that a history of stroke was associated with raised plasma concentrations of CRP (Ford et al 2000, Exel et al 2002). Also, 6 other prospective studies have tested this relationship and their results seem comparable to those on coronary disease (Table 6). The Physicians' Health Study (Ridker et al 1997) and the Honolulu Heart Program (Curb et al 2003) showed that baseline concentration of CRP in apparently healthy men could predict the risk of first ischaemic stroke independently of CVD risk factors. The Cardiovascular Health Study (Cao et al 2003) replicated these results in a population of both men and women. However, in the Framingham Study (Rost et al 2001), raised plasma CRP concentrations independently predicted the risk of future ischaemic stroke in women but not in men. The health ABC study did not find an association between CRP and incident stroke but their follow up period was only 3.6 years (Cesari et al 2003). In a Danish cohort CRP was associated with carotid IMT levels but not with the number of carotid plaques (De Maat et al 2003). Finally, a meta-analysis of studies on stroke with long follow up (>8 years) showed that the relative risk for stroke in healthy individuals with the highest quartile of CRP was 1.68 (95% CI 1.40, 2.01) compared to those with the lowest quartile (Kuo et al 2005).

Notably, despite the wealth of epidemiologic data on CHD, there is only one prospective study on CRP and incident symptomatic PAD which was conducted in a male only population (Ridker et al 1998c). In fact, even cross-sectional data are very limited.

Cassar et al showed that CRP levels are higher in patients with symptomatic PAD (IC or CLI) compared to healthy controls (Cassar et al 2005). Most other published studies have used the $ABI < 0.9$ to define PAD. Among them, the InCHIANTI study (McDermott et al 2005a) and the recent NHANES (Wildman et al 2005) found significant associations between CRP and prevalence of $ABI < 0.9$. However, in a study by McDermott (McDermott et al 2003), CRP was not associated independently with ABI in participants without a history of cardiac or cerebrovascular disease.

Table 6 Prospective studies on CRP and cardiovascular risk in healthy subjects

Study reference	Subjects	Years of follow up	Event	CRP comparisons	Odds ratio or Risk ratio (95% CI)	CVD risk factors adjusted
Kuller et al 1996*	737 men and women	17/ 6-7 [†]	Fatal CHD MI	Top vs. bottom quartile	NR	2.8 (1.4, 5.4)
Ridker et al 1997*	1,086 men	8	MI	Top vs. bottom quartile	NR	0.8 (0.4, 1.7)
Tracy et al 1997*	292 men and women	2.4	Stroke MI	Top vs. bottom quartile	NR	2.9 (1.8, 4.6)
Ridker et al 1998b*	366 women	3	CVD	Top vs. bottom quartile	2.7 (1.04, 6.81)	1.9 (1.1, 3.3)
Ridker et al 1998c*	144 men	5	PAD	Top vs. bottom quartile	4.8 (2.3, 10.1)	NR
Harris et al 1999	675 men and women	4.6	CVD mortality	Top vs. bottom quartile	2.1 (1.0, 4.0)	4.1 (1.7, 9.9)
Koenig et al 1999	936 men	8.2	CHD	1 SD increase	NR	2.2 (1.1, 4.1)
Danesh et al 2000*	1,149 men	9.5	CHD	Top vs. bottom tertile	1.60 (1.23, 2.08) [‡]	1.8 (0.9, 3.6)
Roivainen et al 2000*	482 men and women	8.5	CHD	4 th vs. 1 st and 2 nd quartile	3.46 (2.59, 4.62)	1.50 (1.14, 1.97) [‡]
Packard et al 2000	1,740 men	4.9	CHD	1 SD increase	4.59 (2.68, 7.86)	2.13 (1.38, 3.28)
Gussekloo et al 2000*	165 men and women	5	Stroke (fatal)	Top vs. bottom quartile	1.27 (1.14, 1.42)	3.56 (1.93, 6.57)
Strandberg et al 2000	455 men and women	10	CVD mortality	per 10 mg/L	8.1 (2.5, 26)	1.13 (0.98, 1.29)
Mendall et al 2000	1,239 men	13.7	IHD	Top vs. bottom quintile	1.22 (1.10, 1.35)	NR
Rost et al 2001	591 men 871 women	12-14	Stroke	Top vs. bottom quartile	2.01 (1.14, 3.56)	1.53 (0.59, 2.86)
					2.0 (1.09, 3.78)	1.6 (0.87, 3.13)
					2.9 (1.69, 5.07)	2.1 (1.19, 3.83)

Table 6 cont.

Lowe et al 2001	1,595 men	6.2	IHD	Top vs. bottom quintile	2.49 (1.44, 4.30)	1.60 (0.90, 2.83)
Pirro et al 2001	2,037 men	5.2	CVD	above/ below median	1.8 (1.2, 2.7)	1.0 (0.5, 1.8)
Sakkinen et al 2002	1,717 men and women	20	MI	Top vs. bottom quartile	2.2 (1.6, 3.0)	1.6 (1.1, 2.2)
Pradhan et al 2002b	608 women	2.6	CHD	Top vs. bottom quartile	2.3 (1.4, 3.7)	2.1 (1.1, 4.1)
Ridker et al 2002	27,939 women	8	CVD	Top vs. bottom quintile	4.5 (3.1, 6.6)	2.3 (1.6, 3.4)
Folsom et al 2002*	590 men and women	3.6-4.3	CHD	top vs. bottom quintile	3.3 (2.1, 5.1)	1.5 (0.8, 2.7)
Cesari et al 2003	2,225 men and women	3.6	CHD	Top vs. bottom quartile	NR	1.20 (0.83, 1.75)
Curb et al 2003	1,607 men	20	Stroke	tertile		1.41 (0.73, 2.71)
			Stroke	top vs. bottom quartile	2.3 (1.6, 3.3)	1.6 (1.1, 2.4)
Cao et al 2003	5,417 men and women	10	Stroke	top vs. bottom quartile	1.83 (1.41, 2.36)	1.60 (1.23, 2.08)
Luc et al 2003b *	926 men	5	MI or fatal CHD	Top vs. bottom quartile	NR	2.16 (1.26, 3.72)
Danesh et al 2004	18,569 men and women	12	CHD	top vs. bottom quartile	1.92 (1.68, 2.18)	1.45 (1.25, 1.68)
St-Pierre et al 2005	1,982 men	13	IHD	tertile		
			IHD	top vs. bottom quartile	1.78 (1.22, 2.60)	0.98 (0.65, 1.49)
Cushman et al 2005	3,971 men and women	10	CHD	>3 mg/L vs. <1 mg/L	1.82 (1.46, 2.28)	1.45 (1.14, 1.86)
Lawlor et al 2005	4,286 women	3.5	CHD	1 unit increase	1.09 (1.00, 1.17)	1.03 (0.94, 1.13) [§]
Wilson et al 2005	4,446 men and women	8	CVD	>3 mg/L vs. <1 mg/L	1.60 (1.19, 2.14)	1.22 (0.90, 1.66)
Kistorp et al 2005	765 men and women	5	CVD	1 SD increase	1.26 (1.03, 1.55)	1.17 (0.95, 1.43)

Table 6 cont.

Wakugawa et al 2006	2,692 men and women	12	Stroke	top vs. bottom quintile	NR	3.11 (1.04, 9.32)
Tuomisto et al 2006	7,927 men and women	9	CHD CVD	top vs. bottom quartile	2.83 (1.55, 5.18) 2.62 (1.54, 4.46)	1.90 (0.97, 3.74) 1.73 (0.95, 3.15)

NR: not reported

* nested case-control study

† 17 years for deaths and 6-7 years for MI

‡ hazard ratio, multivariable model adjusted for age and smoking only

|| adjusted for age and smoking only

§ hazard ratio adjusted for cardiovascular risk factors and socioeconomic position

2.6 Interleukin-6

2.6.1 Structure and function

IL-6 is a 26 kDa acute inflammatory cytokine which is produced by several cell types including activated monocytes and macrophages, endothelial cells and adipose cells (Aarden et al 1987; Jirik et al 1989). Its expression is regulated in response to a number of stimuli including endotoxins, IL-1, tumour necrosis factor- α (TNF- α), IL-4 and interferon- γ (Kerr et al 2001).

The biological activities of IL-6 are initiated by binding to a receptor complex. This receptor is made of an 80kDa protein subunit (IL-6R), which binds to the cytokine, and of a 130kDa glycoprotein subunit (gp130), which mediates the signal transduction (Rattazzi et al 2003). Binding of the IL-6/IL-6R complex to gp130 leads to the activation of several transcription factors (Kishimoto et al 1995).

IL-6 has both inflammatory and haemostatic properties. During the acute phase response, by acting on hepatocytes, it up-regulates the production of acute phase proteins. It also has other important inflammatory roles leading to increased endothelial cell adhesiveness by up-regulating E-selectin, ICAM-1, and VCAM-1 and releasing inflammatory mediators, including IL-6 itself (Von der Thusen et al 2003). IL-6 promotes the coagulation cascade through a number of pathways (Kerr et al 2001). It increases the production of platelets and enhances their activation. Also, IL-6 up-regulates several haemostatic variables, including fibrinogen, tissue factor,

vonWillebrand factor (vWF), and factor VIII (Kerr et al 2001;Neumann et al 1997;Stirling et al 1998).

2.6.2 Measurement

IL-6 levels can be measured by bioassays or by enzyme-linked immunosorbant assays (ELISAs). Bioassays usually have low specificity and therefore IL-6 levels are mainly measured in sera and in cell culture supernatants by ELISA, using antibodies against a specific cytokine (Krakauer 1998). ELISA kits are commercially available and they are standardized, generally sensitive, specific and easy to use but can be expensive (Krakauer 1998). In-house ELISAs are commonly used, which makes it difficult to compare them in different laboratories. Their coefficient of variation is generally <15 % (Pearson et al 2003). IL-6 levels are stable in frozen plasma only and IL-6 plasma variability is such that it could be considered acceptable over long periods of time (Rao et al 1994). Dibbs (Dibbs et al 1999) examined the variability of IL-6 in patients with heart failure compared to healthy controls. The authors found that considerable natural, daily, weekly and monthly variability existed both in cases and controls. A circadian variability with increased plasma levels of IL-6 at night in men has also been shown (Sothorn et al 1995).

Increased IL-6 levels have been associated with increased blood pressure, smoking, and diabetes (Rattazzi et al 2003). Also IL-6 levels are increased in obese subjects whereas studies have shown that IL-6 is expressed in adipose tissue (Yudkin et al 2000).

Psychological stress can also elevate circulating levels of IL-6 (Yudkin et al 2000). On the other hand, physical activity is associated with lower plasma levels of IL-6 (Pischon et al 2003). An increase in IL-6 has been described after the menopause and with use of hormone replacement therapy (Davison et al 2003). Finally, its levels have been shown to be influenced by statins (Undas et al 2004).

2.6.3 Interleukin-6 and CVD

IL-6 is much less studied as a cardiovascular risk factor compared to CRP. Table 7 summarizes the prospective epidemiological studies on IL-6 in relation to CVD among healthy individuals. Only 9 prospective studies have been conducted to date with the vast majority of those focusing on coronary events (Cesari et al 2003; Harris et al 1999; Lowe et al 2004b; Luc et al 2003b; Pai et al 2004; Pradhan et al 2002b; Ridker et al 2000a; Ridker et al 2000b). In general, studies showed consistent results suggesting that IL-6 is significantly associated with incident CVD and that after adjustment for cardiovascular risk factors this association retains statistical significance. However, three studies (Pai et al 2004; Ridker et al 2000a; Tuomisto et al 2006) reported non-significant associations between IL-6 and CHD in the multivariable analysis.

IL-6 has also been shown to have significant associations with future CVD in patients with unstable angina, unstable coronary arterial disease, atrial fibrillation and diabetes (Conway et al 2004; Doo et al 2004; Koukkunen et al 2001; Lindmark et al 2001; Pai et al 2004) and it has also been associated with total mortality among patients with CVD

(Volpato et al 2001). Moreover, IL-6 levels are increased in patients with acute MI and also in patients with unstable, but not with stable, angina whereas increased levels of IL-6 and IL-1 receptor antagonist at 48 hours after admission are associated with a complicated in-hospital course (Blake et al 2002; Ikeda et al 2001).

Only the health ABC study has tested the relationship between IL-6 and stroke (Cesari et al 2003). The authors have found significant and independent associations between IL-6 levels and incident stroke. Ridker and colleagues (Ridker et al 2000a) have also included stroke events in their definition of CVD but they did not report specific risk ratio for stroke events only. Results from these 2 studies are in accordance with evidence from several studies that have demonstrated IL-6 increase in serum/plasma of acute stroke patients (Dziedzic et al 2003). On the other hand, in a subsample of the Rotterdam Study (van der Meer et al 2002a), IL-6 levels were found not to be associated with either carotid IMT or carotid plaques. This result was replicated by Chapman (Chapman et al 2004) in a healthy population of 1,111 men and women.

Data on IL-6 and PAD is very limited. In fact, there are no prospective epidemiological data on IL-6 and incident PAD in the general population. Several cross-sectional data exist showing that IL-6 is elevated in patients with PAD compared to healthy controls (Brevetti et al 2003; DePalma et al 2003; Erren et al 1999; McDermott et al 2005a; Signorelli et al 2003; Silvestro et al 2003; Yu et al 2004).

Table 7 Prospective studies on IL-6 and cardiovascular risk in healthy subjects

Study reference	Subjects	Years of follow up	Event	IL-6 comparisons	Odds ratio or Risk ratio (95% CI)	Adjusted for CVD risk factors
Lowe et al 2004b*	1,419 men	4.9	CHD	Top vs. bottom quintile	2.05 (1.43, 2.96)	1.64 (1.11, 2.40)
Luc et al 2003b *	926 men	5	MI or fatal CHD	Top vs. bottom tertile	NR	3.10 (1.77, 5.44)
Pai et al 2004	708 men	8	CHD	Top vs. bottom quintile	1.57 (0.95, 2.57)	1.31 (0.77, 2.22)
Ridker et al 2000a*	794 women				1.92 (1.11, 3.31)	1.05 (0.56, 1.97)
	366 women	3	CVD	Top vs. bottom quartile	2.2 (1.1, 4.3)	0.8 (0.6, 1.1)
Ridker et al 2000b*	404 men	6	MI	Top vs. bottom quartile	2.3 (1.3, 4.3)	2.3 (1.1, 4.6)
Pradhan et al 2002b	608 women	2.9	CHD	Top vs. bottom quartile	3.3 (2.0, 5.5)	2.1 (1.1, 4.0)
Harris et al 1999	675 men and women	4.6	CVD mortality	Top vs. bottom quartile	NR	2.2 (1.0, 4.8)
Cesari et al 2003	2,225 men and women	3.6	CHD	Top vs. bottom tertile	NR	1.61 (1.09, 2.38)
			Stroke			3.70 (1.07, 8.21)
St-Pierre et al 2005	1,982 men	13	IHD	top vs. bottom quartile	2.28 (1.52, 3.43)	1.71 (1.07, 2.75) [†]
Tuomisto et al 2006	7,927 men and women	9	CHD	top vs. bottom quartile	1.54 (0.88, 2.68)	1.10 (0.57, 2.14)
			CVD		1.72 (1.05, 2.84)	1.25 (0.69, 2.23)

* nested case-control study

[†] adjusted for CVD risk factors and for fibrinogen and CRP

2.7 Adhesion molecules

Adhesion molecules are transmembrane glycoproteins that mediate cell-cell and cell-extracellular matrix interactions. They can be divided into a number of families based on their function and structure including the selectins, the immunoglobulin superfamily and the integrins (Hope et al 2003).

2.7.1 Structure and function

E-selectin

E-selectin is a member of the selectin family and is expressed by activated endothelial cells. Selectins are glycoproteins with a conserved structure containing an N-terminal C-type lectin domain, an epidermal growth factor like domain and a variable number of short consensus repeat domains, a short transmembrane domain and finally a small cytoplasmic domain (Blankenberg et al 2003). E-selectin mediates the first step in leukocyte adhesion at sites of inflammation or injury and it is involved in the initiation of rolling and tethering of leukocytes to the endothelial surface, to platelets, or to other leukocytes (McEver et al 1995). Also, it interacts with ligands and creates weak bonds between activated endothelial cells or leukocytes. Finally, it favours interactions between platelets and leukocytes (Kansas 1998).

ICAM-1 and VCAM-1

The immunoglobulin superfamily includes glycoprotein receptors with immunoglobulin like domains that are active in various tissues. The intracellular adhesion molecule-1

(ICAM-1) and the vascular adhesion molecule-1 (VCAM-1) are the most common members of this family. ICAM-1 is widely expressed at a basal level and is up-regulated by pro-inflammatory cytokines in leukocytes and endothelial cells. It mediates adhesion of leukocytes to activate endothelium and participates in leukocyte extravasation (Blankenberg et al 2003). VCAM-1 is expressed on endothelial cells and participates in the leukocyte's firm adhesion to the endothelial wall. It also triggers endothelial cells to change their shape allowing the emigration of leukocytes in the arterial wall. Expression of E-selectin, ICAM-1 and VCAM-1 is induced by inflammatory cytokines mainly by TNF- α and IL-1 β and maximum cell surface expression observed after 4-6 hours (Blankenberg et al 2003).

The aforementioned adhesion molecules may be discarded from the endothelial cell surface and found in soluble forms in the peripheral blood by a mechanism which is little understood (Ridker 2001). Circulating forms of adhesion molecules are thought to be the result of a shedding/ proteolytic mechanism which rapidly removes adhesion molecules from the cell surface following endothelial cell activation (Leeuwenberg et al 1992). Consequently, these soluble forms measured in the plasma are considered to be direct measures of the expression of membrane molecules.

2.7.2 Measurement

Levels of soluble adhesion molecules in the plasma may be measured by ELISA techniques. However, the availability of commercial kits is generally limited,

standardization does not exist yet and little is known about their pre-analytical conditions (Roberts 2004). Their levels are unstable unless frozen. Their coefficient of variation is estimated <15% (Pearson et al 2003).

Circulating levels of adhesion molecules have been associated with hormone replacement therapy (Blankenberg et al 2003). Also their levels have been shown to be increased in people with diabetes, hypertension or increased cholesterol levels and in smokers (Blankenberg et al 2003). Some studies have also shown associations with statin therapy but others did not confirm those results (Hope et al 2003). Finally, studies have found associations between ICAM-1 and E-selectin with BMI or the presence of clinical obesity (Hope et al 2003).

2.7.3 Adhesion molecules and CVD

Adhesion molecules have received considerable attention in epidemiologic research as risk factors for CVD. Increased levels have been associated with future events in patients with pre-existing disease. Blankenberg et al. (Blankenberg et al 2001) reported that baseline soluble VCAM-1, ICAM-1, and E-selectin were all significantly related to future death from cardiovascular causes among 1,246 patients with documented CAD. In support, Wallen et al. (Wallen et al 1999) showed that patients with stable angina pectoris who developed an MI or died from cardiovascular causes had elevated serum levels of soluble cell adhesion molecules, ICAM-1, VCAM-1 and E-selectin. Yin et al.

(Yin et al 2003) showed that levels of ICAM-1 and VCAM-1 were related to clinical outcomes among subjects with coronary heart failure.

Several prospective studies that have been conducted in healthy populations are listed on Table 8. A combined analysis of the 5 studies on ICAM-1 yielded an odds ratio of 1.39 (1.11, 1.73) for CHD in those in the top third compared with those in the bottom third of baseline measurements (Malik et al 2001). For VCAM-1 and E-selectin, the combined analysis yielded an odds ratio of 1.02 (0.81, 1.29) and 1.16 (0.87, 1.55), respectively (Malik et al 2001). The Physicians' Health Study, the ARIC study, the Prospective Epidemiological Study of Myocardial Infarction (PRIME) study and a British cohort study, found significant associations between ICAM-1 and incident CHD which were independent of cardiovascular risk factors (Hwang et al 1997; Luc et al 2003a; Malik et al 2001; Ridker et al 1998a). Only, in the Cardiovascular Health Study, ICAM-1 failed to show significant associations in the multivariable analysis (Jenny et al 2006). Finally, Malik (Malik et al 2001), showed that ICAM-1 was no longer significantly associated with incident CHD when the analysis was further adjusted for markers of socioeconomic status.

Compared to ICAM-1, VCAM-1 and E-selectin have shown consistently negative results in relation to CHD. Five prospective studies on VCAM-1 failed to show significant associations between VCAM-1 and incident CHD even in unadjusted analysis (Table 8). E-selectin was only investigated by two prospective studies but showed no evidence for associations in the multivariable analysis (Table 8).

As with IL-6, the relationship between adhesion molecules and stroke events is under-studied compared to CHD events. Ridker (Ridker et al 2000a) also included stroke events in their definition of CVD but they did not report the odds ratio explicitly for stroke events. The PRIME study reported the unadjusted odds ratio (95% CI) for stroke per 1SD increase of ICAM-1 levels as 1.07 (0.92, 1.27) (Jenny et al 2006). In contrast, the ARIC study reported increased risk for carotid artery disease among those with increased ICAM-1 (OR (95% CI) for 1 SD increase 1.38 (1.12, 2.72)) and E-selectin (1.71 (1.13, 1.75)) but not for those with increased VCAM-1 levels independently of CVD risk factors (Hwang et al 1997). Finally, among people with CHD, elevated concentrations of ICAM-1 were associated with increased risk of ischaemic stroke, independent of other traditional cerebrovascular risk factors and of plasma fibrinogen (Tanne et al 2002).

Further evidence for associations between stroke and adhesion molecules exist from cross-sectional data and post stroke studies. In fact, acute ischaemic stroke was associated with elevated plasma levels of ICAM-1, VCAM-1 and E-selectin, independent of age, sex and other recognized risk factors for stroke (Simundic et al 2004). Also, the serum level of ICAM-1 on admission was associated with neurological deterioration of ischaemic stroke (Wang et al 2006). In support, Rohde (Rohde et al 1998) reported a strong correlation between carotid artery IMT and plasma ICAM-1 and VCAM-1. Also, ICAM-1 was independently associated with the risk of having at least one carotid plaque and with the risk of having at least one femoral plaque but not with IMT in another study (Bongard et al 2002). In contrast to these studies, it was found that

symptomatic carotid disease was not associated with increased expression of adhesion molecules in the endothelium of advanced carotid plaques or in the circulation (Nuotio et al 2003). Finally among subjects who had experienced stroke or who had asymptomatic carotid stenosis, ICAM-1 levels were not associated with ischaemic events after more than 3 years of follow up (Ehrensperger et al 2005).

In relation to PAD even fewer data exist and studies are usually small, use different diagnostic criteria for PAD and are conducted in different population groups (Hope et al 2003). One prospective study, examined the association between ICAM-1 and VCAM-1 with symptomatic PAD. ICAM-1, and not VCAM-1, was associated with a more than a 3-fold increase in the risk of developing symptomatic PAD in analysis adjusted for cardiovascular risk factors (Pradhan et al 2002a). Both increased ICAM-1 and VCAM-1 were found in patients with PAD compared to healthy controls (Blann et al 1998). Peter and colleagues (Peter et al 1997) found that VCAM-1 was associated with the presence and the extent of PAD assessed by extensive angiography. To date, there are no prospective data on E-selectin. In a case-control study, Signorelli (Signorelli et al 2003) showed that increased E-selectin levels were found among those with IC. However, two other studies failed to show differences in circulating levels of E-selectin between people with and without PAD (Blann et al 1994;De Caterina et al 1997)

Table 8 Prospective studies on adhesion molecules and cardiovascular risk in healthy subjects

Study reference	Population	Years of follow up	Event	Level comparisons	Odds ratio or Risk ratio (95% CI)	
					Crude or Age adjusted	Adjusted for CVD risk factors
ICAM-1						
Malik et al 2001*	1,921 men	16	CHD	Top vs. bottom tertile	1.68 (1.32, 2.14)	1.49 (1.14, 1.94)
Ridker et al 1998a*	948 men	9	MI	Top vs. bottom quartile	1.6 (1.1, 2.4)	1.8 (1.1, 2.8)
Ridker et al 2000a*	366 women	3	CVD	Top vs. bottom quartile	2.6 (5.3, 2.1)	1.1 (0.8, 1.4)
Hwang et al 1997*	792 men	5	CHD	1 SD increase	1.97 (1.56, 2.43)	1.92 (1.47, 2.50)
Luc et al 2003a*	951 men	5	CHD	Top vs. bottom tertile	2.45 (1.64, 3.65)	2.09 (1.34, 3.24)
Jenny et al 2006*	2,049 men and women	10	MI	1 SD increase	1.12 (0.97, 1.30)	1.16 (0.99, 1.36)
			Stroke		1.07 (0.92, 1.27)	1.03 (0.87, 1.21)
			CVD		1.14 (0.98, 1.31)	1.15 (0.99, 1.35)
Pradhan et al 2002a*	280 men	9	mortality	Top vs. bottom quartile	3.9 (1.7, 8.6)	3.2 (1.4, 7.4)
VCAM-1						
Malik et al 2001*	1,921 men	16	CHD	Top vs. bottom tertile	1.26 (0.99, 1.61)	0.96 (0.66, 1.40)
de Lemos et al 2000	948 men	9	MI	Top vs. bottom quartile	1.28 (0.85, 1.92)	1.17 (0.71, 1.91)
Hwang et al 1997*	792 men	5	CHD	1 SD increase	1.03 (0.86, 1.24)	0.98 (0.77, 1.25)
Luc et al 2003a*	951 men	5	CHD	Top vs. bottom tertile	0.81 (0.57, 1.16)	0.89 (0.61, 1.32)
Pradhan et al 2002a*	280 men	9	PAD	Top vs. bottom quartile	1.3 (0.6, 2.5)	1.3 (0.6, 2.7)

Table 8 cont.

E-selectin					
(Malik et al 2001)*	1,921 men	16	CHD	Top vs. bottom tertile	1.27 (1.00, 1.61) 1.13 (0.78, 1.62)
(Hwang et al 1997)*	792 men	5	CHD	1 SD increase	1.54 (1.27, 1.86) 1.19 (0.93, 1.53)

* nested case-control study

2.8 Fibrinogen

2.8.1 Structure and function

Fibrinogen is a plasma glycoprotein with molecular weight of 40 KDa (Doolittle et al 1998). It consists of three pairs of polypeptide chains, two A α , two B β , and two γ which are bond together by disulphide bonds. The composition of fibrinogen varies according to differences in amino acids residues in the three polypeptide chains. The most common variants are due to A α chain heterogeneity. Among them three forms have been identified, a high molecular weight fibrinogen and two low molecular weight fibrinogen's variants (Mosesson 1983).

Fibrinogen is synthesized in the liver by hepatocytes and has a biological half life of approximately 100 hours (Kamath et al 2003). It circulates in the plasma at concentrations between 1.5g/L and 4.5g/L whereas concentrations above 0.5g/L are required for normal haemostasis. Fibrinogen is the main coagulation protein in plasma. In the final step of the coagulation cascade, it is transformed into fibrin under the action of thrombin. In addition, as CRP, fibrinogen is an acute phase reactant. Hepatic synthesis of fibrinogen is regulated by inflammatory cytokines, mainly by IL-6. There is also evidence that IL-6 production is dependent on prior stimulation by fibrin degradation products.

2.8.2 Measurement

Fibrinogen is relatively unstable and plasma should be frozen as soon as possible if not analyzed immediately (Roberts 2004). There are two main methods for determining fibrinogen levels, the 'functional' and the 'direct' method. The first is, in essence, measuring the coagulation time which is proportional to the fibrinogen concentration. The Clauss method which is the most commonly used functional method records the time taken to reach the formation of a clot (Kamath et al 2003). The disadvantage of this method is that it is associated with a degree of variability due to heterogeneity of fibrinogen structure and also due to other haemostatic factors (such as fibrin degradation products) that can become attached to the clot and alter the clotting time. In addition, the Clauss method can underestimate the fibrinogen concentrations in the presence of hirudin peptide 54-65 and fibrinogen fragment E (De Cristofaro et al 1998; Roberts 2004).

The direct methods quantify fibrinogen either gravimetrically, immunologically or by precipitation (Kamath et al 2003). In the first method thrombin is added to a sample of plasma and allowed to set until the clot is formed. Fibrinogen is then measured by gravimetry. This assay is, in general, time consuming and laborious. On the contrary, immunological assays are easy to use and do not take much time. They involve ELISA methods that use monoclonal antibodies that recognize the A α chain but do not recognize other fibrinogen derivatives. Finally, in precipitation assays fibrinogen is precipitated (by heat or salting out) and then measured usually by nephelometry. In this method, total fibrinogen is measured and therefore the estimated fibrinogen is generally

higher than that estimated by the functional assays. However, other molecules such as fibrin degradation products can be precipitated and then measured leading to overestimated fibrinogen levels.

The imprecision of most fibrinogen assays ranges between 4% and 8% (Roberts 2004). The Clauss method has a larger methodological variability than other fibrinogen assays (Rosenson et al 2001). The precipitation- nephelometric assay has shown a methodological coefficient of variation between 2.4 and 3.0% whereas the gravimetric method has a reported a coefficient of variation of 1% (De Maat et al 1996;Krobot et al 1992). The methodological coefficient of variation for fibrinogen with the Clauss method was 3.3 to 4.5% when an automated procedure was used that averaged the results from three samples each assayed in duplicate (Rosenson et al 2001).

Due to the large number of assays proposed for the determination of fibrinogen, the standardization is problematic. A 2nd international plasma standard (WHO 2nd International Standard [IS] Fibrinogen, Plasma 98/612) is available for fibrinogen which uses the Clauss method (Whitton et al 2000). This standard has been established at 2.19 g/L of clottable fibrinogen.

In epidemiologic research different fibrinogen assays have been used. A systematic review did not show heterogeneity between studies that used different fibrinogen assays (Danesh et al 1998). The Caerphilly and Speedwell studies directly compared a precipitation-nephelometric assay and a Clauss method assay for the prediction of

ischaemic heart disease (IHD) (Sweetnam et al 1998). They concluded that the direct method was a significantly stronger predictor of IHD than the clotting method (Sweetnam et al 1998). Moreover, the correlation coefficient between the two measurements was only 0.62 (Yarnell et al 1985).

Finally, several factors that influence fibrinogen levels have been described. In summary, raised fibrinogen is associated with older age, female sex, smoking, obesity, reduced physical activity, diabetes, hypertension and increased cholesterol levels (Kamath et al 2003). Moreover, menopause, oral contraception and stress have been also associated with high fibrinogen levels (Koenig 2003; Lee et al 1993; Meade et al 1983). On the other hand, decreased fibrinogen has been associated with moderate alcohol consumption and with hormone replacement therapy (Koenig 2003; Lee et al 1993). Fibrinogen levels also show seasonal variation with higher levels in winter than in summer (Stout et al 1991).

2.8.3 Fibrinogen and CVD

Since the first report by Meade (Meade et al 1983), the role of fibrinogen in CVD prediction has been extensively studied in epidemiological studies. All now agree that fibrinogen is an independent predictor of CHD in healthy subjects and in subjects with pre-existing CVD. Less evidence is available on stroke and PAD. Table 9 summarizes the prospective epidemiological studies that have examined the relationship between fibrinogen and CVD in healthy populations. The results on CHD are remarkably

consistent and with very few exceptions provide support for fibrinogen as a strong and independent predictor of CHD (Table 9). Meta-analysis of published studies of plasma fibrinogen on CHD showed a risk ratio of 1.8 (1.6, 2.0) between the top vs. the bottom tertile of plasma fibrinogen. A recent individual patient meta-analysis of 7,213 cases confirmed this result (Fibrinogen Studies Collaboration 2005). The age and sex adjusted hazard ratio (95% CI) per 1g/L increase in fibrinogen level for CHD was 2.42 (2.24, 2.60); stroke, 2.06 (1.83, 2.33); other vascular mortality, 2.76 (2.28, 3.35); and non-vascular mortality, 2.03 (1.90, 2.18). The hazard ratios for CHD and stroke were reduced to about 1.8 after adjustments for established cardiovascular risk factors which was further reduced after adjustment for intra-individual variability in confounders (Fibrinogen Studies Collaboration 2005).

Lind (Lind et al 2001) investigated the interaction between fibrinogen and other inflammatory proteins. Among 6,075 men, they observed that any other marker of inflammation (orosomucoid, α_1 -antitrypsin, haptoglobin, and ceruloplasmin) in addition to increased fibrinogen was associated with a further increase in incident CHD and CVD mortality compared to fibrinogen alone. Another study proposed that the effect of fibrinogen varied according to the atherogenic lipid profile (Assmann et al 1996). When fibrinogen and LDL levels were considered together, there was a graded and dramatic 8-fold increase in 8 year risk from 17 per 1,000 of population among men with both fibrinogen and LDL cholesterol in the lower tertiles to 130 per 1,000 in men with both of these parameters in the upper tertile (Assmann et al 1996). In support, results from Cantin (Cantin et al 2002) showed an interaction between fibrinogen and Lp(a) in CVD

risk prediction. Finally, fibrinogen has been associated with future CHD among high risk populations with diagnosed diabetes, IC, angina or CHD (Koenig 2003).

As with other biomarkers studied here, the relationship between fibrinogen and stroke is much less studied compared to that of CHD (Table 9). However, few studies are consistent in showing an independent association between increased fibrinogen and stroke events which is comparable to that observed in CHD (Smith et al 2005; Smith et al 1997; Wilhelmsen et al 1984). Further evidence supports these results showing that fibrinogen levels remaining elevated after stroke and were associated with an increased risk of a recurrent vascular event (Beamer et al 1998). Also, in patients with a previous transient ischaemic attack or ischaemic stroke, risks of recurrent ischaemic stroke and acute coronary events increased linearly with fibrinogen levels (Rothwell et al 2004). In addition, in 473 first-ever ischaemic stroke patients fibrinogen in the top tertile within the first 24 hours after stroke onset was associated with a 63% excess risk of new a cardiovascular event compared to patients in the bottom tertile (Di Napoli et al 2002).

Moreover, fibrinogen was associated with carotid plaque formation independently of cardiovascular risk factors (Chapman et al 2004; De Maat et al 2003). In support of these findings, in a population sample of adults without clinically overt atherosclerotic disease, elevated fibrinogen was related to carotid IMT independent of a wide range confounding variables (Martinez-Vila et al 2003). Fibrinogen was also associated with IMT in the ARIC study but not in the Glostrup study (De Maat et al 2003; Folsom et al 1993).

The association between fibrinogen and PAD has been confirmed by many cross-sectional studies (Fabsitz et al 1999;McDermott et al 2003;McDermott et al 2005a;Meijer et al 2000;Selvin et al 2004;Smith et al 1993;Wildman et al 2005;Zheng et al 2005). However, evidence from prospective studies is very limited. The prognostic value of fibrinogen was illustrated in the Edinburgh Artery Study, in which median fibrinogen levels were higher in patients who later went on to develop PAD (Smith et al 2000b). These results were confirmed by the Physicians' Health Study (Ridker et al 2001).

Table 9 Prospective studies on fibrinogen and cardiovascular risk in healthy subjects

Study reference	Population	Years of follow up	Event	Fibrinogen comparisons	Odds ratio/ risk ratio (95% CI) or (SD)	Crude/ Age adjusted	Adjusted for CVD risk factors
Meade et al 1986	1,494 men	10	IHD	1 SD increase	1.50 (1.23, 1.84)	1.50 (NR)	1.39 (NR)
Wilhelmsen et al 1984	792 men	13.5	MI		NR (p=0.01)	NR (p=0.15)	NR (p=0.15)
Kannel et al 1987	554 men	12	Stroke		NR (p=0.01)	NR (p=0.05)	NR (p=0.05)
	761 women		CVD				
Yarnell et al 1991	4,860 men	5.1	Stroke	Top vs. bottom quintile	4.1 (2.6, 6.5)		2.6 (1.6, 4.3)
		3.2	IHD				
Heinrich et al 1994	2,044 men	6	CHD	Top vs. bottom tertile	NR (p<0.001)		NR (p=0.05)
Rosengren et al 1996	664 men	9	CHD	Top vs. bottom tertile	2.5 (1.2, 5.2)		1.5 (0.7, 3.4)
Folsom et al 1997	6,297 men	5.2	CHD	Top vs. bottom tertile	1.76 (1.48, 2.10)		1.48 (1.19, 1.84)
	8,180 women				1.54 (1.36, 1.75)		1.21 (1.02, 1.44)
Cremer et al 1997	5,790 men	10	MI	Top vs. bottom quintile	3.4 (2.3, 4.9)		NR
Smith et al 1997	1,592 men and women	5	MI	1 unit increase	1.24 (1.08, 1.42)		1.44 (1.13, 1.84)
			Stroke		1.04 (0.89, 1.22)		1.52 (1.17, 1.98)
Woodward et al 1998	3,930 men	8	CVD	Top vs. bottom quintile	3.01 (1.66, 5.45)		2.32 (1.23, 4.38)
	3,760 women		mortality		3.42 (0.98, 11.87)		1.90 (0.48, 7.50)
Tracy et al 1999	5,888 men and women	5	CVD	1 SD increase	1.27 (1.12, 1.44)		1.16 (1.02, 1.33)
					1.24 (1.02, 1.33)		1.13 (0.99, 1.29)
Ma et al 1999*	14,916 men	5	MI	Top vs. bottom quintile	NR		1.50 (0.77, 2.90) [‡]
Smith et al 2000b	1,080 men and women	5	PAD	1 unit increase	1.40 (1.10, 1.77)		1.35 (1.05, 1.73)
Cooper et al 2000	1,153 men	7.8	CHD	1 log SD increase	1.45 (1.19, 1.76)		1.31 (1.06, 1.63)
Sato et al 2000	11,977 men and women	4.8	CHD	Top vs. bottom	6.1 (1.8, 20.9)		4.8 (1.4, 16.8)

Table 9 cont.

	women	MI	quartile		
Lowe et al 2000	6,595 men	CHD	1 SD increase	4.9 (1.4, 17.2)	3.8 (1.1, 13.4)
Ridker et al 2001*	280 men	PAD	Top vs. bottom quartile	1.33 (1.21, 1.47)	1.12 (1.00, 1.25)
Yano et al 2001	3,571 men	CVD mortality	1SD increase	2.3 (1.2, 4.7)	2.2 (1.1, 4.7)
Salomaa et al 2002	2,378 men and women	CHD	1 SD increase	1.40 (1.26, 1.55)	1.18 (1.03, 1.36)
Cantin et al 2002	2,215 men	CHD	Top vs. bottom tertile	1.16 (0.93, 1.46)	0.99 (0.77, 1.26)
Palmieri et al 2003	2,671 men and women	CVD	10 mg/dL increase	2.46 (1.44, 4.20)	1.55 (1.18, 1.78)
Smith et al 2005	2,398 men	CVD	Top vs. bottom tertile	NR	1.03 (1.01, 1.05)
St-Pierre et al 2005	1,982 men	Stroke	Top vs. bottom quartile	1.70 (1.36, 2.14)	1.32 (1.04, 1.67)
		CHD	1 SD increase	1.59 (1.00, 2.52)	1.51 (0.93, 2.45)
		IHD	Top vs. bottom quartile	1.74 (1.34, 2.27)	1.26 (0.96, 1.66)
			1 SD increase	2.29 (1.52, 3.45)	1.53 (0.97, 2.43) [§]
Rajecki et al 2005	2,372 men and women	Stroke	1 SD increase	1.13 (0.91, 1.40)	1.07 (0.86, 1.32)
		CVD	1 SD increase	1.38 (1.19, 1.58)	1.22 (1.05, 1.41)

NR: not reported

* Nested case control study

† These are standardised coefficients of logistic regression analysis. CI were not reported.

‡ adjusted for age and smoking only.

|| stepwise regression, fibrinogen, total cholesterol, systolic blood pressure and age were included in the model.

§ Adjusted for CVD risk factors and CRP and IL-6.

2.9 Coagulation factors

Several markers that reflect the ongoing coagulation process can be measured in plasma samples. Here, 3 major markers of coagulation, factor VII, vWF and prothrombin fragments 1+2 (F1+2), which have been analysed in epidemiological research, are described.

2.9.1 Structure and function

Factor VII

Factor VII is a vitamin K dependent serine protease with a pivotal role in coagulation since it initiates the extrinsic coagulation cascade. In this form, it is a 406 amino acid residue glycoprotein with a molecular mass of about 50kDa and a plasma half-life of 5 ± 6 hours. Factor VII is converted to its active form factor VIIa in the presence of tissue factor. By binding to tissue factor it converts factor IX and X into their active forms and leads to thrombin formation (Feinbloom et al 2005). The majority of factor VII in blood is in the form of inactivated factor VII zymogen, while trace amounts of factor VIIa are present. The activation of zymogen factor VII to enzyme factor VIIa is largely dependent on tissue factor and arises at the site of vascular injury after formation of a TF/FVII complex (Eigenbrot 2002). The key event in the activation of factor VII is a cleavage of the peptide bond between Arg15 and Ile16 (Eigenbrot 2002).

vWF

Previously named factor VIII related antigen, vWF, is a multimeric glycoprotein secreted by the endothelium. It comprises of 50-100 monomers and is subject to post-transcriptional modification before becoming functional (Blann 2006). Different molecular weight multimers of vWF exist (Wagner 1990). Also, there are two release pathways of vWF, constitutive and regulated. Constitutively secreted vWF is predominately composed of small multimers (Lip et al 1997). However, regulated release occurs from preformed intracellular storage granules (Weiber-Palade bodies) and they consist of high molecular weight polymers that are most effective in mediating platelet binding (Blann 2006). The main function of vWF is to carry factor VIII in the circulation. Through this role it also regulates the formation of fibrin thrombus at the site of the injury. Under normal circumstances, plasma vWF is derived from endothelial cells rather than from platelets making vWF a good marker of endothelial dysfunction.

F1+2

Another marker of the coagulation process, F1+2, is released during the conversion of prothrombin to thrombin in the presence of factor Xa. Its plasma life is approximately 90 minutes. Therefore levels of F1+2 are a marker of thrombin formation and can be used as a marker of this in vivo (Saigo et al 2004). The formation of thrombin from prothrombin requires cleavage of two peptide bonds in the prothrombin molecule. Prothrombin consists of a single polypeptide chain of nearly 608 amino acids and can be described as having two halves—a “pro” half and a “thrombin-forming” half (Becker et al 1998). The amino terminal end (pro half), which has been designated prothrombin

1+2, contains approximately 50% of the molecular mass (35,500 Da) (Becker et al 1998).

2.9.2 Measurement

Factor VII can be measured in several ways including measuring zymogen mass, factor VII activity or factor VII antigen. The classic assay which measures factor VII coagulation activity (factor VIIc) is based on the ability of a test plasma to correct the clotting time of FVII deficient plasma (Marckmann et al 1998). This is an one-stage clotting assay in which the clotting time is recorded and factor VIIc is expressed based on a reference plasma calibration curve. The factor VIIc measurement is dependent on the quality of the factor VII deficient plasma (Poggio et al 1991). The active factor VIIa can be measured by an ELISA assay based on factor VIIa antibodies or by a clotting assay which uses a truncated, soluble, recombinant TF that possesses cofactor function for FVIIa (Marckmann et al 1998). Finally, the total factor VII protein concentration in plasma may be assessed by two methods with comparable results, either by an ELISA assay or by a functional two-stage assay.

Similarly, plasma vWF can be measured in various ways. The vWF antigen assay is probably the best tool for measuring the level of total vWF. However, it detects all vWF multimer forms without discrimination and it does not assess vWF function (Favaloro et al 2002). Functional assays such as the VWF:RCo provide a marker of vWF function and allow the quantification of intermediate and high molecular weight vWF multimer

forms. A 5th international standard for vWF antigen and VWF:RCo has recently been established (Hubbard et al 2004). Alternatively, vWF:Multimer testing identifies the structural integrity of vWF, the retention or loss of various molecular weight forms of vWF, and qualitative vWF defects. Nevertheless, it does not give quantitative information on detected vWF and is expensive and time consuming (Favaloro et al 2002).

F1+2 is measured by standard immunoassays (ELISA). In general, the pre-analytical and analytic properties of F1+2 are very little studied. The assay has low sensitivity and it has a relatively low susceptibility to in vitro artefacts.

Both F1+2 and vWF are elevated in pregnancy (Fareed et al 1998). Moreover, factor VII concentrations have also increased with serum glucose, blood pressure, BMI, and post menopausal status in women (Pearson 1997). Similarly, vWF is associated with smoking, BMI, physical activity, blood pressure, alcohol and cholesterol levels (Yarnell et al 2000). No circadian variation on vWF levels has been found. Plasma levels of vWF antigen have also been found to be lower in persons of blood group O compared to those in non-O groups (Meade et al 1994). Finally, F1+2 has been associated with smoking, high levels of triglyceride, and low levels of glucose (Cushman et al 1996); however, the ARIC study did not report any associations between F1+2 levels and cardiovascular risk factors (Folsom et al 2001).

2.9.3 Coagulation factors and CVD

There is substantial epidemiologic research on coagulation markers and CVD. Table 10 lists prospective epidemiologic studies in the general population on vWF, factor VII and F1+2 and incident CVD. vWF has received considerable attention whereas F1+2 has been little studied. Also, as with previous markers, the vast majority of studies focus on coronary atherosclerosis. The only meta-analysis available is for vWF: 5 prospective studies with 1,524 CHD cases yielded a combined risk ratio (95% CI) of 1.5 (1.1, 2.0) in individuals with vWF in the top third compared with those in the bottom third (Whincup et al 2002). However, most individual studies up to date, demonstrated that vWF often loses statistical significance in the multivariable analysis and that the magnitude of the reported risk ratios is generally modest.

On the other hand, most prospective studies in patients with baseline evidence of arterial disease show independent associations of vWF with future vascular events (Cortellaro et al 1993;Jansson et al 1991;Thompson et al 1995). For example, in the ECAT study among 3,043 patients with angina pectoris, those with levels of vWF in the top quintile compared to independently had a subsequent 20% increase in the risk of acute coronary syndrome after 2 years of follow up (Thompson et al 1995).

A meta-analysis has not been reported on either factor VII or on F1+2 and CHD. Table 10 lists the prospective epidemiological studies on these markers and incident CVD in the general population. The first Northwick Park Heart Study demonstrated an association between factor VII activity and incident CVD but only in univariable

analysis (Meade et al 1993). Surprisingly, two studies, the Second Northwick Park Study and the Caerphilly Prospective Study found an inverse significant association in multivariable analysis between factor VII and CVD (Cooper et al 2000; Smith et al 2005). Although the authors suggest that this might be due to chance, the fact that both studies had the same conclusions merits further investigation. Almost all the other studies provided no evidence for an association between factor VII and CVD (Table 10). The same is true for F1+2 because cohort studies, including the ARIC, the Second Northwick Park Study and the Caerphilly Prospective Study, did not show statistically significant associations between this marker and incidence of CVD. However, conflicting results have been reported from studies on subjects with pre-existing CVD and from cross-sectional analyses. F1+2 was elevated in those with angiographically verified coronary atherosclerosis as compared to patients with normal coronary arteries (Giannitsis et al 1999). Similarly, factor VII was higher in those who had experienced an MI compared to healthy controls and in people with angiographically defined coronary stenoses (Pearson et al 1997).

None of the three markers has been associated with incident stroke. However, F1+2 levels (median 1.28 vs. 1.06 nmol/L) and vWF levels (216 vs. 198 IU/dL) levels were higher in patients with progressive stroke than in stable/improving patients (Barber et al 2004). In addition, levels of vWF were elevated initially (1.86 IU/ml) and after 3 months (1.51 IU/ml) in stroke patients compared with a healthy reference population (1.26 IU/ml) (Catto et al 1997). In the same study, analysis of 6 month case fatality, vWF

levels were associated with risk of death in stroke patients (odds ratio 1.73 (1.12, 2.66) for an increase of 1 U/ml), even after allowing for stroke type (Catto et al 1997).

There are very few data on these markers and PAD in the literature. After 5 years of follow up in the Edinburgh Artery Study, factor VII and vWF showed no significant associations with incident PAD (Smith et al 2000b). Supporting these results, a case-control study showed that vWF and factor VII were not significantly increased in individuals with PAD defined by an ABI<0.9 (Philipp et al 1997).

Table 10 Prospective studies on coagulation factors and cardiovascular risk in healthy subjects

Study reference	Population	Years of follow up	Event	Levels comparisons	Odds ratio or Risk ratio (95% CI)	
					Crude/ Age adjusted	Adjusted for CVD risk factors
Factor VII						
Meade et al 1993	1,511 men	5	CVD mortality	1SD increase	NR	1.34 (1.08, 1.60)
Smith et al 1997	1,592 men and women	5	IHD	1 unit increase	1.02 (0.85, 1.22)	0.98 (0.83, 1.15)
Folsom et al 1997	6,297 men	5.2	Stroke		1.23 (0.87, 1.76)	1.08 (0.73, 1.59)
Junker et al 1997	8,180 women		CHD	1 SD increase	1.06 (0.92, 1.22)	1.01 0.86-1.18
	2,780 men	8	CHD		1.18 (1.02, 1.37)	0.99 0.81-1.23
Smith et al 2000b	1,080 men and women	5	PAD	1 SD increase	NR (p=0.02)	NR (p=0.04)
Tracy et al 1999	3,336 men	5	CHD	Top vs. bottom quintile	1.14 (1.03, 1.27)	NR (p>0.05)
Cooper et al 2000	2,455 women				NR (p>0.05)	NR (p>0.05)
	1,097 men	7.8	CHD	1 SD increase	1.08 (0.86, 1.36)	0.58 (0.46, 0.71)
Smith et al 2005	2,398 men	13	CVD	Top vs. bottom tertile	0.82 (0.62, 1.09)	0.71 (0.52, 0.96)
			CHD		0.78 (0.57, 1.08)	0.65 (0.46, 0.92)
			Stroke		0.96 (0.56, 1.67)	0.91 (0.50, 1.68)
Rajecki et al 2005	2,372 men and women	9	CVD	1 SD increase	1.07 (0.91, 1.26)	0.94 (0.80, 1.11)
F1+2						
Folsom et al 2001*	1,018 men and women	6	CHD	Top vs. bottom quintile	0.67 (0.4, 1.2)	0.80 (0.4, 1.6)
Smith et al 2005	2,398 men	13	CVD	Top vs. bottom tertile	1.08 (0.80, 1.40)	1.01 (0.75, 1.38)
			CHD		0.93 (0.52, 1.69)	0.96 (0.52, 1.75)
			Stroke		1.13 (0.80, 1.60)	1.03 (0.72, 1.48)

Table 10 cont.

Salomaa et al 2002	2,240 men and women	6	CHD	1 SD increase	1.26 (1.00, 1.58)	1.19 (0.93, 1.51)
Cooper et al 2000 vWF	1,097 men	7.8	CHD	1 SD increase	1.08 (0.90, 1.29)	NR
Meade et al 1994	1,393 men	16.1	IHD			
Folsom et al 1997	6,297 men 8,180 women	5.2	CHD	1 SD increase	1.19 (1.06, 1.34)	1.05 (0.91, 1.20)
Smith et al 1997	1,592 men and women	5	IHD	1 unit increase	1.18 (1.00, 1.38)	1.02 (0.84, 1.25)
Thogersen et al 1998*	156 men and women	1.5	Stroke MI	Top vs. bottom quartile	1.09 (0.93, 1.28)	0.95 (0.81, 1.12)
Jager et al 1999	610 men and women	5	CVD	Top vs. bottom tertile	1.27 (0.95, 1.72)	1.15 (0.85, 1.57)
Rumley et al 1999	2,223 men	5	mortality IHD	Top vs. bottom quartile	2.39 (1.05, 5.44)	2.58 (0.87–7.63)
Smith et al 1999	1,080 men and women	5	PAD	1 SD increase	2.80 (1.18, 6.66)	3.08 (1.20, 7.91)
Whincup et al 2002*	1,891 men	16	CHD	Top vs. bottom tertile	1.22 (1.03, 1.44)	1.20 (1.01, 1.43)
Johansson et al 2002	324 men and women	11	Ischaemic Stroke	Above vs. below median	1.23 (1.02, 1.47)	1.17 (0.97, 1.41)
Morange et al 2004*	832 men	5	CHD	Top vs. bottom quartile	1.36 (1.05, 1.76)	1.31 (0.99, 1.72)
Smith et al 2005	2,398 men	13	CVD CHD Stroke	Top vs. bottom tertile	1.55 (1.16, 2.09)	1.59 (1.17, 2.18)
					1.01 (0.61, 1.67)	NR
					NR	3.04 (1.59, 5.80)
					1.12 (0.89, 1.42)	1.06 (0.84, 1.35)
					1.18 (0.90, 1.56)	1.09 (0.83, 1.45)
					0.98 (0.62, 1.56)	0.97 (0.61, 1.56)

* nested case control study

NR: not reported

2.10 Fibrinolytic factors

In addition to coagulation factors, a number of molecules that participate in fibrinolysis may serve as measurements of thrombin and plasmin formation. This section is focused on three pivotal molecules of the fibrinolytic process, t-PA, D-dimer and fibrinopeptide A (FpA).

2.10.1 Structure and function

t-PA

t-PA regulates fibrinolysis by activating the conversion of plasminogen to plasmin. It is synthesized and secreted by endothelial cells and is a single chain enzyme with molecular weight of 68kD (Dobrovolsky 2002). Only a small amount of t-PA is active whereas the remaining is bound to its inhibitor PAI-1. The complex t-PA/PAI-1 retains affinity to fibrin and therefore competes with free t-PA in the binding of fibrin. Thus, free plasma levels of t-PA should be a marker of fibrinolytic activity (Saigo 2004).

D-dimer

The digestion of fibrinogen yields fragments X, Y, D and E which are known as fibrin degradation products (Fareed 1998). The digestion of fibrin yields distinct fragments products including D-dimer which consists of two fibrin monomers, cross linked through $\gamma\gamma$ chain. Thus plasma levels of D-dimer reflect the extent of cross-linked fibrin turnover without significant interference with fibrinogen or non-cross linked fibrin degradation products (Saigo 2004, Lowe 1995).

FpA

Finally, when thrombin cleaves fibrinogen in the A α and B β chain, fibrinopeptides A (FpA) and B (FpB) are released, respectively. FpA is initially released more rapidly than FpB, but soon the rate of FpB release accelerates (Binnie et al 1993). FpA is a 16 amino acid peptide with a molecular weight of 1540 D and a very short plasma half-life (3-5 min) (Stegnar et al 2006). Since the fibrinogen molecule has a dimeric structure, two identical FpA molecules can be cleaved from each fibrinogen molecule (Stegnar et al 2006). Thrombin, in turn, cleaves fibrinopeptides leading to fibrin monomers that spontaneously polymerize to form a fibrin clot. Therefore levels of FpA are specific for thrombin activity and the formation of fibrin (Fareed et al 1998).

2.10.2 Measurement

The mean plasma levels of t-PA are 5-10 ng/ ml and its half life is 2-3 min (Dobrovolsky 2002). The assessment of t-PA is complicated by a number of factors. PAI-1 rapidly binds to t-PA to inhibit its functional activity. Thus, it is difficult to perform functional assays for t-PA unless steps (such as acidification of the plasma) are taken to inhibit the interaction between t-PA and PAI-1. Alternatively, the plasma concentration of t-PA can be determined by immunologic assays, such as enzyme-linked immunosorbent assays, for which there are commercially available reagents. Thus the measured active t-PA reflects three processes, the secretion of t-PA from endothelial cells, the inhibition of t-PA by PAI-1 and other molecules, and the clearance of t-PA by the liver (Chandler et al 2000).

A variety of different methods are available for the measurement of D-dimer. These include manual latex agglutination assays, ELISAs, latex-enhanced photometric immunoassays (LPIA), and membrane-based immunofiltration assay (IFA) systems (Dempfle 2001). However, the numerical results of these assays vary noticeably. Two attempts have been made to standardize D-dimer assays, but the researchers concluded that the standardization of D-dimer is impossible and so reference preparations and/or guidelines have not been distributed (Gaffney 1995, Nieuwenhuizen 1997).

Dempfle (Dempfle 2001) evaluated 23 quantitative D-dimer assays and concluded that they differ concerning specificity for cross-linked fibrin, and preference for detecting either high molecular weight fibrin complexes, or low molecular weight fibrin degradation products. These results were also confirmed by an external quality assessment program (De Maat 2000). However, Meijer (Meijer et al 2006) recently developed a model for harmonization between different quantitative D-dimer methods. This harmonization resulted in a reduction of the variability between method-specific consensus values from 75% to approximately 25% (Meijer et al 2006).

Normal FpA plasma levels are around or below 2 ng/mL. Sensitive methods for FpA measurement are radioimmunoassay or enzyme-linked immunoassay (ELISA), however, these have very long turn-around times (Stegnar et al 2006). In addition, sample handling of FpA requires careful acquisition, collection, and processing in order to avoid thrombin elaboration and ex vivo increases in FpA concentration (Stegnar et al 2006). Alternatively, urine measurement of FpA can be more sensitive.

t-PA has shown diurnal variation with elevated levels in the morning (Andreotti et al 1991;Jafri et al 1992). Also, D-dimer and t-PA have been associated with cardiovascular risk factors such as BMI, smoking, diabetes, alcohol, blood pressure, total and HDL cholesterol (Stegnar et al 2006). Associations between cardiovascular risk factors and FpA are less consistent (Cushman et al 1996). There is also some evidence for associations between these fibrinolytic factors with hormone replacement therapy (Koh 2002;Pinto et al 1997;Sowers et al 2005).

2.10.3 Fibrinolytic factors and CVD

Fibrinolytic factors have been investigated in several epidemiologic studies in relation to CVD. A recent meta-analysis on D-dimer of 7 population studies with 1,535 CHD cases found a significant odds ratio (95% CI) of 1.7 (1.3, 2.2) for individuals in the top vs. those in the bottom tertile at baseline (Danesh et al 2001). This association is of similar strength to that between plasma fibrinogen and CHD. Similarly, when 7 available prospective cohort studies on t-PA and CHD in general populations (2,119 cases) were synthesized, the combined odds ratio was 2.2 (1.8, 2.7) after adjustment for age and sex only, which decreased to 1.5 (1.2, 1.8) after further adjustments for cardiovascular risk factors (Lowe et al 2004a).

To date, at least 15 prospective studies among apparently healthy individuals of either D-dimer or t-PA have been conducted, as listed in Table 11. Most studies suggest that D-dimer levels are associated with incident CHD independently of cardiovascular risk factors although the estimated relative risks were modest. Interestingly, in an elderly

cohort, D-dimer was a better predictor of events early in the follow up than later in the follow up (Cushman et al 1999). In the same study, D-dimer had stronger associations among those with subclinical disease. Moreover, in the West of Scotland Coronary Prevention Study, D-dimer showed a significant independent relationship with coronary risk even when it was adjusted for other inflammatory molecules including fibrinogen, CRP and IL-6 (Lowe et al 2004b).

There is less evidence for an association of t-PA with CVD. In fact, in most studies, adjustment for potential confounders seems to reduce considerably the strength of the associations usually below statistical significance (Lowe et al 1998; Thogersen et al 1998). Pradhan (Pradhan et al 2004) evaluated both t-PA and D-dimer and found that elevated t-PA antigen and, to a lesser extent, D-dimer were independently associated with incident coronary events among postmenopausal women. In the same cohort, the odds ratio for women with baseline elevations in both t-PA antigen and D-dimer was greater than for those with low levels of both biomarkers or with elevations of either marker alone (Pradhan et al 2004). Only one epidemiological study has examined the associations between FpA and incident cardiovascular events in a male cohort (Cooper et al 2000). The authors found no evidence for an association with CHD events (Table 11).

D-dimer and t-PA have also been associated with recurrent events in people with existing coronary disease (Moss et al 1999; Niessner et al 2003). They have also been shown to predict cardiovascular events in people with pre-existing PAD (Smith et al 1998). Elevated D-dimer levels have also been shown to be an independent risk factor

for cardiovascular mortality in patients with heart failure (Alehagen et al 2004). t-PA has shown strong associations with future CHD in people with angina in two other studies (Juhan-Vague et al 1996;Thompson et al 1995). Increased levels of FpA have also been documented in patients with unstable angina who developed acute MI or who were at increased risk of cardiovascular mortality (Ardissino et al 1996;Li et al 1999). D-dimer and FpA are also elevated in people with atrial fibrillation (Kumagai et al 1990).

Data on fibrinolytic markers and stroke is very limited. Two prospective studies have shown strong and independent associations between D-dimer and stroke events with more than a 2 fold increase in the risk of stroke for people in the highest tertile of D-dimer (Smith et al 2005;Smith et al 1997). Similarly, 4 prospective studies have shown comparable results for t-PA (Johansson et al 2000;Ridker et al 1994b;Smith et al 2005;Smith et al 1997). The Caerphilly Prospective Study failed to show independent associations between t-PA and incident stroke (Smith et al 2005). Nevertheless, the Physicians' Health Study, the Edinburgh Artery Study and the MONICA study reported strong independent associations between t-PA and future stroke events (Ridker et al 1997b; Smith et al 1997; Johansson et al 2000).

D-dimer has also shown prognostic information after ischaemic stroke (Barber et al 2006) and along with FpA has been independently associated with survival after stroke (Feinberg et al 1996). Elevated levels of D-dimer and FpA were also reported following brain infarction (Kataoka et al 2000). However, both D-dimer and t-PA failed to show associations with recurrent stroke in two other studies (Barber et al 2006;Squizzato et al

2006). In addition, there is evidence that D-dimer levels might be associated with stroke subtype. After acute stroke, D-dimer levels were significantly higher in the cardioembolic group than in the atherothrombotic and lacunar groups (Ageno et al 2002).

D-dimer and t-PA have not shown evidence for associations with IMT (De Maat et al 2003; Lee et al 1998). However, in the ARIC study both markers were higher in subjects with IMT in the top compared to the bottom tertile (Salomaa et al 1995). In a Finnish male cohort, FpA correlated with common carotid and carotid bifurcation IMT but only in the univariable and not in the multivariable analysis (Rankinen et al 1994). These associations were abolished after adjusting for confounders (Rankinen et al 1994).

The relationship between these fibrinolytic markers and PAD is not well-established. In the Edinburgh Artery Study, t-PA and D-dimer were not associated with incident PAD after 5 years of follow up (Smith et al 2000b). However, both plasma markers have shown higher levels with the presence and increasing severity of PAD in cross-sectional analyses (Cassar et al 2005; Killewich et al 1998; Smith et al 1995; Unlu et al 2006). Data on FpA is even more limited. In cross-sectional analysis of the Edinburgh Artery Study, FpA was not significantly elevated in subjects with PAD but prospective data was not reported (Lowe et al 1993).

Table 11 Prospective studies on fibrinolytic factors and cardiovascular risk in healthy subjects

Study reference	Population	Years of follow up	Event	Levels comparisons	Odds ratio or Risk ratio (95% CI)	
					Crude/ Age adjusted	Adjusted for CVD risk factors
D-dimer						
Ridker et al 1994a	592 men	5	MI	95 th vs. 5 th percentile	2.02 (1.04, 4.02)	2.12 (1.05, 4.28)
Smith et al 1997	1,592 men and women	5	IHD	1 unit increase	1.31 (1.00, 1.71)	1.04 (0.78, 1.37)
			Stroke		2.16 (1.31, 3.55)	1.96 (1.12, 3.41)
Lowe et al 1998	1,998 men	5	MI	Top vs. bottom quintile	4.22 (2.23, 7.98)	3.53 (1.82, 6.85)
			CHD mortality			
Cushman et al 1999*	292 men and women	2.4	CHD	Top vs. bottom quartile	3.1 (1.1, 8.6)	4.1 (1.2, 14.5)
Folsom et al 2001*	1,018 men and women	6	CHD	Top vs. bottom quintile	1.89 (1.1, 3.3)	4.21 (1.9, 9.6)
Smith et al 2000b	1,080 men and women	5	PAD	1 unit increase	0.79 (0.48, 1.28)	0.72 (0.43, 1.18)
Lowe et al 2001	1,690 men	6.2	IHD	Top vs. bottom quintile	1.68 (1.00, 2.82)	1.15 (0.65, 2.05)
Danesh et al 2001*	1,899 men	16	CHD	Top vs. bottom tertile	1.67 (1.31, 2.13)	1.79 (1.36, 2.36)
Lowe et al 2004b	3,213 men	7.5	IHD	Top vs. bottom quintile	2.17 (1.52, 3.09)	1.76 (1.18, 2.64)
Pradhan et al 2004	608 women	2.9	CHD	Top vs. bottom quartile	2.0 (1.2, 3.2)	1.7 (1.0, 2.9)
Smith et al 2005	2,398 men	13	CVD	Top vs. bottom tertile	1.84 (1.42, 2.37)	1.60 (1.23, 2.06)
			CHD		1.75 (1.30, 2.35)	1.45 (1.07, 1.98)
			Stroke		2.14 (1.27, 3.62)	2.09 (1.23, 3.56)

2.11 Summary

Inflammation and haemostasis are two interrelated and universal responses to injury.

Recent advances in molecular biology have suggested that these two mechanisms also play an important role in atherosclerotic development and progression. Molecular markers representing activated inflammation and haemostasis might therefore serve as attractive measurements of the ongoing atherosclerotic process.

Epidemiological research has focused on several markers and tested their associations with CVD. This thesis focuses on 12 markers of inflammation and haemostasis in relation to atherosclerotic disease and in particular to peripheral atherosclerosis. Markers of systemic inflammation under study include CRP, the proinflammatory cytokine IL-6, and soluble adhesion molecules such as ICAM-1, VCAM-1 and E-selectin. CRP has received the most attention and, along with IL-6, appears to be a consistent predictor of future cardiovascular events in large prospective studies. Epidemiological data on adhesion molecules predicting risk of CVD is less consistent. ICAM-1 has shown independent associations with CHD in prospective studies but VCAM-1 and E-selectin have shown consistently negative results.

For the haemostatic markers, fibrinogen has been studied extensively and overwhelming evidence that it is an independent predictor of CHD in healthy subjects and in subjects with pre-existing CVD exists. D-dimer, t-PA and vWF have shown less consistent

evidence whereas factor VII, FpA and F1+2 have shown very little or no evidence for associations with CVD.

Overall, the epidemiological research on these molecules in relation to stroke or especially PAD is sparse. Some evidence for associations between CRP and fibrinogen with stroke and PAD exist but very little data is available on the other biomarkers.

Chapter Three

3 Aim and Objectives

Chapter 1 highlighted the fact that PAD is a relatively common disease especially when asymptomatic individuals are accounted for. However, very little data is available on incident asymptomatic PAD and the risk factors associated with its progression. In addition, the literature review in Chapter 2 demonstrated that several markers of inflammation and haemostasis have been hypothesized as potential risk factors for CHD in numerous studies but their relation with PAD is not well-established and for many of them unknown.

3.1 Aim

The aim of this thesis is to determine whether plasma levels of several inflammatory and haemostatic markers are related to the progression of PAD and to the incidence of PAD events in order to identify possible mechanisms involved in the aetiology, development and progression of disease. For this reason, the Edinburgh Artery Study, a population cohort study which is described in detail in the following chapter, was used. The inflammatory markers, CRP, IL-6, ICAM-1, VCAM-1 and E-selectin, and the haemostatic markers, fibrinogen, D-dimer, t-PA, vWF, factor VII, FpA and F1+2 were examined.

3.2 Objectives

The main endpoints examined here were: progression of peripheral atherosclerosis and incidence of clinical PAD. Similar analyses were performed for both endpoints. More specifically, the objectives of the analyses were:

3.2.1 Progression of peripheral atherosclerosis

1. To examine the change in a non-invasive marker of subclinical peripheral atherosclerosis, the ABI, over 5 and 12 years of follow up in the general population.
2. To determine univariable and multivariable associations between traditional cardiovascular risk factors (age, sex, BMI, diabetes, total/ HDL cholesterol ratio, smoking, physical activity) and progressive peripheral atherosclerosis measured by the change in ABI after 5 and 12 years of follow up.
3. To determine univariable associations between inflammatory markers (CRP, IL-6, ICAM-1, VCAM-1, E-selectin) and progressive peripheral atherosclerosis measured by the change in ABI after 5 and 12 years of follow up.
4. To determine univariable associations between haemostatic markers (fibrinogen, D-dimer, t-PA, vWF, factor VII, F1+2, FpA) and progressive peripheral atherosclerosis measured by the change in ABI after 5 and 12 years of follow up.
5. To determine multivariable associations between each of the aforementioned inflammatory or haemostatic marker with progressive peripheral atherosclerosis

measured by the change in ABI after 5 and 12 years of follow up accounting for traditional cardiovascular risk factors.

6. To determine multivariable associations between the aforementioned inflammatory and haemostatic marker with peripheral progressive atherosclerosis measured by the change in ABI after 5 and 12 years of follow up after accounting for each other.
7. To compare the relative associations of the aforementioned inflammatory and haemostatic marker and examine whether they have an additive effect on the progression of peripheral atherosclerosis measured by the change in ABI after 5 and 12 years of follow up.
8. To examine the associations of the aforementioned inflammatory or haemostatic markers with progression of peripheral atherosclerosis measured by the change in ABI after 5 and 12 years of follow up according to cardiovascular risk factor status.

3.2.2 Incident PAD

1. To examine the incidence of clinical PAD, not only of IC but also of the more severe form of CLI (rest pain, gangrene, ulceration and surgical intervention).
2. To determine univariable associations between inflammatory markers (CRP, IL-6, ICAM-1, VCAM-1, E-selectin) and incident PAD over 12 years of follow up.

3. To determine univariable associations between haemostatic markers (fibrinogen, D-dimer, t-PA, vWF, factor VII, F1+2, FpA) and incident PAD over 12 years of follow up.
4. To determine multivariable associations between each of the aforementioned inflammatory or haemostatic marker with incident PAD over 12 years of follow up after accounting for traditional cardiovascular risk factors.
5. To determine multivariable associations between each of the aforementioned inflammatory and haemostatic marker after accounting for each other with incident PAD over 17 years of follow up.
6. To compare the relative associations of the aforementioned inflammatory and haemostatic marker and examine whether they have an additive effect on incident PAD over 17 years of follow up.
7. To examine the associations of the aforementioned inflammatory or haemostatic markers with incident PAD over 17 years of follow up according to cardiovascular risk factor status.

3.2.3 Incident CVD, MI and stroke

Apart from progression of peripheral atherosclerosis and incident PAD another endpoint of combined cardiovascular events (MI and stroke) was also included in the following analysis. This analysis had the following objectives:

1. To determine if the previously described associations between inflammatory and haemostatic markers and PAD occurred with other manifestations of atherosclerosis including MI and stroke.
2. To examine if any of the inflammatory and haemostatic markers were more important for specific cardiovascular endpoints (peripheral, coronary or cerebrovascular).
3. To determine univariable associations between inflammatory markers or haemostatic markers and incident stroke after 17 years of follow up.
4. To determine multivariable associations between inflammatory markers or haemostatic markers and incident stroke after accounting for traditional risk factors.
5. To determine multivariable associations between inflammatory markers or haemostatic markers and incident CVD, MI or stroke after accounting for a measure of subclinical atherosclerosis (ABI).

Chapter four

4 Methods

4.1 Introduction

Here, the methodology of this thesis is described. As it was already mentioned, the analysis is based on the Edinburgh Artery Study: a population based cohort which was designed to study risk factors for CVD. The study began in 1987 and took place in the Department of Community Health Sciences, University of Edinburgh and was headed by Professor Gerry Fowkes. It was funded by the British Heart Foundation. At the end of this chapter the statistical methodology is outlined.

4.2 Study design

The Edinburgh Artery Study commenced as a cross-sectional survey in 1987 and continued as a longitudinal study. The target population for the Edinburgh Artery Study comprised inhabitants of Edinburgh aged 55-74 years old. A random sample stratified by 5 years age band was selected from 10 general practises serving a wide range of socioeconomic and geographical areas throughout the city. The sample size of 1,500 participants was estimated on the basis of the number required to conduct a subsequent follow up study with adequate power to detect differences in incidence of vascular events according to baseline characteristics. The overall recruitment summary is shown in Figure 1. In order to produce at least 1,500 participants, 272 subjects were selected

from each practice: 34 males and females from each 5 years age band. General practitioners reviewed lists of their patients selected for the study and excluded those unfit to participate (e.g. due to mental illness), those who had moved from the practice, or those who had died. These exclusions were replaced by other randomly selected patients.

Following publicity in the local media, letters of invitation signed jointly by the study director and a partner in each general practice were sent to the subjects inviting them to attend a university clinic for a medical examination. Transport or an examination at home was available for those having difficulty in attending the clinic. Travelling expenses were offered. Letters returned by the Post Office were replaced with invitations to other patients selected at random. On receipt of an affirmative reply, an appointment was booked and a map, along with details of the examination was sent. Responders who did not attend were offered a second appointment usually by telephone. Non-responders were sent a second letter of invitation.

Some 20% (222 subjects) of non-responders in each practice were randomly selected and followed up. Each was sent a letter and enclosing a short questionnaire. Subjects not returning the questionnaire were telephoned or visited at home on up to three occasions at different times of the day and evening. Thirty-seven had moved address on their general practitioner register, 6 had died or were in the hospital and 9 could not be contacted, 17 refused to take part and 152 completed the questionnaire by post, phone or

home visit. The follow up of the non-responders did not show any particular bias (Figure 4). The study was approved by the Lothian Health Board Ethics Committee and informed consent was obtained from each participant.

Figure 3 Recruitment summary of the Edinburgh Artery Study

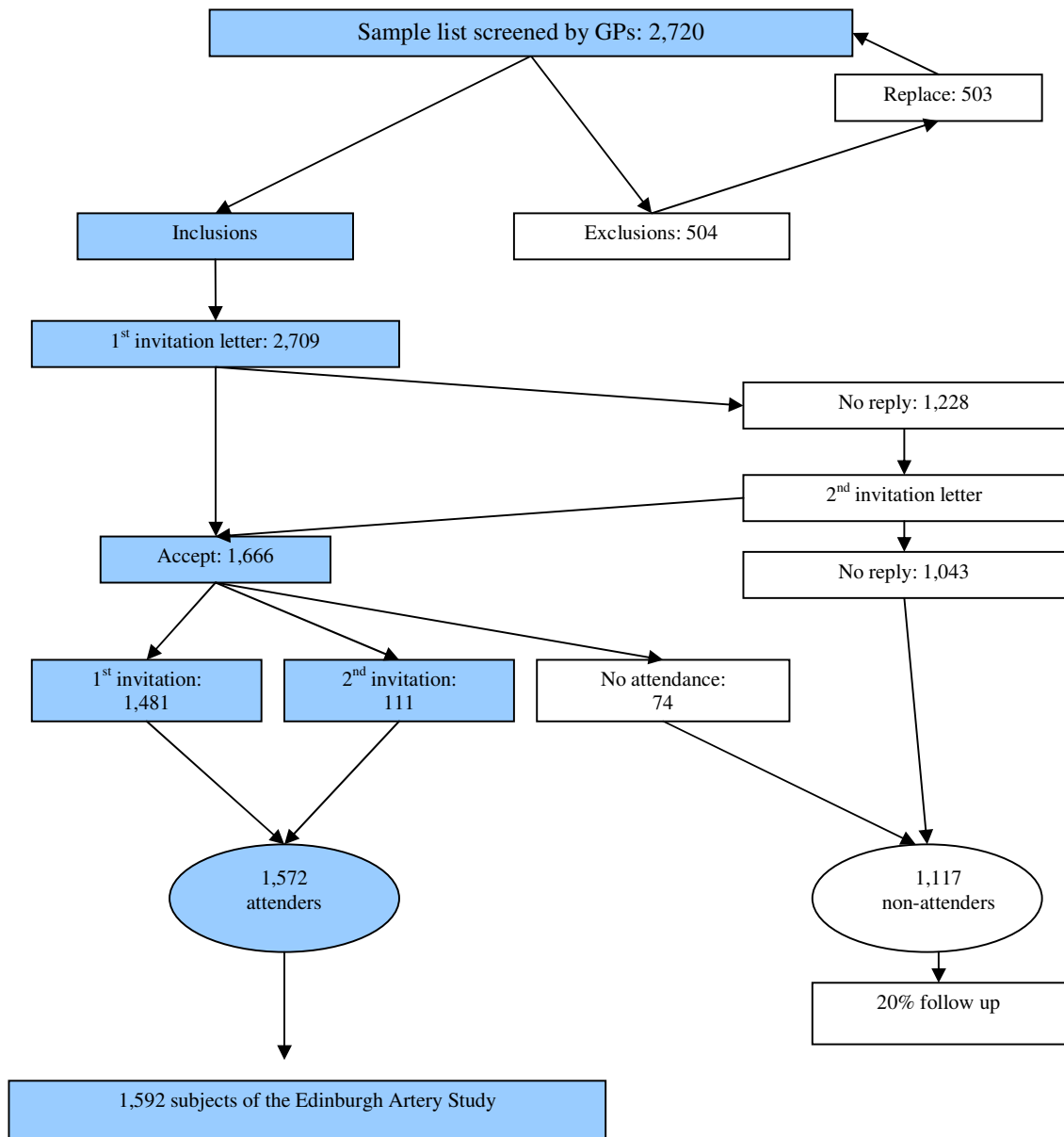
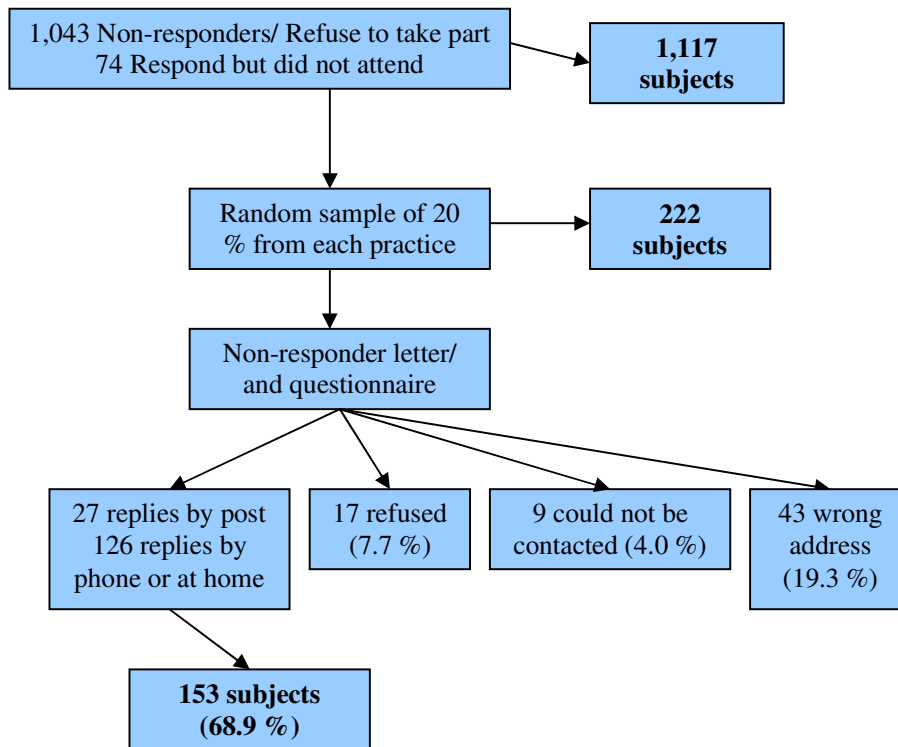


Figure 4 Follow up of the non-responders/ non attenders



4.3 Five and 12 years follow up

Follow up examinations were held after 5 and after 12 years from the baseline examination. During both follow up examinations, participants still residing in the Edinburgh area were sent a letter of invitation asking if they wished to attend the second/ third examination, and if so, whether they would travel to the clinic (expenses offered) or if they would like to be examined at home. Participants who lived outside the area were offered overnight accommodation and travel expenses but not a visit at home. Those who did not wish to attend, missed appointments or did not respond after three invitations were sent a self-administrative questionnaire. Participants whose questionnaire was not returned were first telephoned and then visited at home and asked to complete the questionnaire. Finally, participants whose letters were returned by the Post Office were traced through Lothian Health Board and if a new address was found the invitation procedure was repeated.

4.4 Clinical examinations

At baseline, clinical examinations were held each weekday morning from August 1987 to September 1988. The 5 year follow up examination was held between November 1992 and March 1999; whereas, the 12 year follow up examination was held between February 1999 and October 2001.

Before conducting the main study at baseline, a pilot study was carried out on 50 volunteers from one general practice. The quality of measurement were checked before and during the study by repeat measurements taken intermittently by the study

co-ordinator. Individual observer measurements were assessed for drift. Variability of some measurements especially ABI measurement was previously assessed (Fowkes et al 1998).

Standing height (without shoes) was measured to the nearest 5mm using a free standing metal ruler on a heavy base. Weight (without shoes and outer clothing) was measured to the nearest 100g on digital scales (Soehnle). BMI was calculated as the weight in kilograms divided by square of the height in meters. A 12-lead ECG was taken and coded independently by two observers using the Minnesota code (Prineas et al 1982). If a discrepancy occurred, a final decision was made by a consultant cardiologist. Systolic and diastolic (phase V) blood pressures were recorded in the right arm only, after 10 minutes rest in the supine position, using a Hawksley random zero sphygmomanometer. The femoral, posterior tibial, and dorsalis pedis pulses were palpated in both legs. Ankle systolic pressures were measured first in the right leg and then in the left leg at the posterior tibial artery, using a Sonicaid Doppler ultrasound probe and a random zero sphygmomanometer with the cuff placed proximal to the malleoli. The pulse was located with the Doppler probe, and the cuff was inflated until the pulse was no longer audible. The cuff was then deflated, and the pressure was noted when the pulse re-appeared. If the posterior tibial pulse was not detectable, the dorsalis pedis pulse was used wherever possible. The ABI was calculated by dividing the ankle systolic pressure by the brachial systolic pressure. The lower of the two leg indices was used in the analysis as indicative of worse disease.

4.5 Blood processing and laboratory assays

At baseline, 20 ml of venous blood and a urine sample was taken initially for subsequent estimation of biochemical, haemostatic and inflammatory factors. All samples were taken between 09:00am and 12:30pm to minimise diurnal variation. Subjects were asked to refrain from smoking for two hours prior to the visit and to fast from 11:00pm the previous evening if not diabetic.

4.5.1 Glucose

A fasting blood sample was taken for measurement of blood glucose and then each subject not known to be diabetic consumed 75g of glucose in the form of 335ml Solripe Gluctoza Health Drink (Strathmore Mineral Water Company, Forfar, Scotland). A second blood glucose specimen was taken 2 hours after the oral glucose load. Both fasting and post-glucose samples were analysed by the hexokinase U.V. method using a standard kit (Boehringer).

4.5.2 Serum Lipids

Tests for serum total cholesterol and high density lipoprotein (HDL) cholesterol were performed on a Cobas Bio analyzer (Roche Products) using standard kits. Total cholesterol was measured using an analysis kit from Boehringer which is an enzymatic colometric test. Magnesium chloride and dextran sulphate were used to precipitate the VLDL and LDL protein fractions. The HDL cholesterol was estimated in the supernatant using the Boehringer cholesterol kit.

4.5.3 Inflammatory and haemostatic markers

The assays for the inflammatory and haemostatic markers except for fibrinogen were carried out in the Haemostasis, Thrombosis and Vascular Medicine Unit at the Glasgow Royal Infirmary. Fibrinogen was measured at the University of Edinburgh. For these assays, all blood samples were centrifuged within 2 hours of collection and were stored the same day at -40°C. Storing time varied between variables from 5 years (haemostatic markers) to 10 years (inflammatory markers). Each variable was assayed for the whole population at the same time. In addition, internal quality control plasma was run for each marker and measurements of markers that fell outside the 95% normal range were repeated.

CRP was measured immunologically using a high-sensitivity assay in a BN ProSpec nephelometer (Dade Behring, Milton Keynes, UK). Plasma levels of IL-6, ICAM-1, VCAM-1, E-selectin, were measured using high sensitivity ELISA kits (R&D Systems, Oxford, UK). The coefficients of variation were as follows, respectively: for CRP, 4.7%; for IL-6, 7.4%, for ICAM-1, 4.8%; for VCAM-1 4.3% and for E-selectin, 4.8.

vWF, D-dimer and t-PA were measured using high sensitivity ELISA kits (DAKO (Copenhagen, Denmark); AGEN (Parsippany, NJ, USA) and Biopool (Umea, Sweden); respectively). Fibrinogen was measured in citrated plasma by a thrombin-clotting turbidometric method in a centrifugal analyser. Urinary FpA was measured by radioimmunoassay (IMCO, Stockholm); factor VII was measured using a chromogenic assay (Kabi Diagnostica); and plasma F1 + 2 was measured by ELISA

(Dade Behring, Marburg, Germany). The coefficients of variation were as follows, respectively: for fibrinogen, 5.2%; for D-dimer, 12.7%, for t-PA, 9.3%; for vWF, 7.2%; for factor VII, 8.1; and for F1+2, 9.2.

Data on some markers was missing due to attrition or decreasing availability of plasma sample. This was mostly due to the volume of the original sample and is not expected to be associated with any of the demographic and cardiovascular risk profile of the study subjects. Thus, it was considered as missing 'at random'. To demonstrate this, Table 12 shows the cardiovascular risk factors of the cohort in subjects without and with missing CRP. As expected, the numbers fluctuated slightly between the two groups but no major differences were observed. Same was true for the other markers included in the analysis.

Table 12 Baseline characteristics in people with and without C-reactive protein (CRP) measurement at baseline

	Subjects with CRP measurements		Subjects with missing CRP measurement	
	N	Mean (SD)/ Percent	N	Mean (SD)/ Percent
Age (years)	1,154	64.8 (5.67)	438	64.8 (5.72)
Males	570	51 %	239	54 %
BMI (kg/m ²)	1,154	25.6 (3.91)	437	25.2 (3.70)
Total cholesterol (mmol/L)	1,154	7.03 (1.33)	419	6.97 (1.29)
HDL cholesterol (mmol/L)	1,151	1.44 (0.41)	416	1.41 (0.42)
Total/ HDL cholesterol ratio	1,151	5.24 (1.96)	416	5.45 (2.81)
Pack years smoking [†]	1,129	23.9 (4.12)	431	24.3 (4.41)
Diabetes	69	6 %	22	5 %
Hypertension	629	55 %	241	55%
Cardiovascular disease				
Myocardial infarction	26	2.8 %	13	3.0 %
Stroke	16	1.7 %	9	2.1 %
Angina	80	6.9 %	27	6.2 %
Intermittent claudication	38	4.1 %	15	4.3 %
Any of the above	192	16.6 %	85	19.4 %
Physical activity				
Light	446	38.7 %	146	33.3 %
Moderate	463	40.2 %	211	48.2 %
Strenuous	126	10.9 %	41	9.4 %
Ankle brachial index	1,145	1.03 (0.17)	434	1.02 (0.18)

[†] among smokers

4.6 Self-administrative questionnaire

At baseline, 5 and 12 years follow up, subjects completed a self administrative questionnaire which contained mostly validated questions about personal characteristics, social class (Office of Population Censuses and Surveys 1980), medical history, smoking, exercise, personality, alcohol consumption and diet. Medical history items addressed subject's recall of a diagnosis by the doctor of stroke or MI. Chest pain, angina and leg pain symptoms were assessed by the WHO angina and WHO IC questionnaires, respectively (Appendix I) (Rose 1962). Smoking habits were self-reported and information was converted into pack years for each person obtained by calculating the number of 20-cigarette packs per day smoked, multiplied by number of years being a smoker. This measure was judged to be sufficiently accurate because the self-reported levels correlated with thiocyanate levels. Information on diabetes status, use of insulin injections and tablets for diabetes was also recorded

4.7 Follow up of cardiovascular events

Information about the following cardiovascular events was obtained during the follow up period (approximately 17 years): MI, angina, stroke, transient ischaemic attacks, IC, CLI, thrombo-embolism, vascular surgery, angioplasty and coronary artery bypass grafting (CABG). Data was collected from 5 main sources:

- a) Scottish National Health Service Central Registry
- b) Information and Statistics Division of the Common Services Agency

- c) Annual questionnaires
- d) General Practitioners
- e) Hospital Records

4.7.1 Scottish National Health Service Central Registry

To identify the cardiovascular events each study participant was flagged at the United Kingdom National Health Service Central Registry, thus ensuring any deaths certificates would be automatically forwarded for all subjects dying within the UK. The name, address, date of birth, sex and study number of each patient was typed onto an index card and forwarded to the Central Registry. When notification of death was received by the Central Registry, the death certification was sent (after a period of at least 2 months) to the University of Edinburgh Medical School. The primary and secondary causes of death were recorded on the database in a file restricted to deceased patients. When a post-mortem was conducted information was used to verify the underlying cause of death. All causes of death had been allocated a diagnostic code according to the ISD-9 or ISD-10. In addition, the cause of death was allocated a code indicating whether the patient had died of MI (code 1), stroke (code 2), other CVD (code 3), or non-cardiovascular causes (code 4).

4.7.2 Information and Statistics Division

To obtain information on the non-fatal events, information was sought from the Information and Statistics Division (ISD) of the Scottish Office Home and Health Department. The ISD office holds patient data derived from summaries created when

patients are discharged from hospitals. An application was sent to the ISD at the beginning of the study to obtain access to the Scottish Morbidity Records on all patients participating in the study. The procedure entailed record linkage to computerised ISD data with our own patient data. The merged file was then released in the form of a computerised print-out with the cases in a specific order. The ISD subsequently provided a computer printout at several time points during the follow up identifying all subjects who had attended a hospital for a vascular event according to specific ICD codes, in addition to the relevant hospital and date of submission and discharge.

4.7.3 General Practitioners

At the start of the study, information on general practices and doctors was obtained from each participant. A letter was then sent to each listed doctor explaining the purpose of the study along with a card with participant's details. The doctor was then asked to attach the card to the front of the subject's records and to return it after a cardiovascular event or if patient had died. A pre-paid envelope was also sent for the reply. Also, the card had to be returned if the subject had changed address or general practice in which case they were traced by contacting the Practitioner Services. If a new general practice was found, it was contacted and a pink card along with a cover letter was sent to them. When a card was returned to notify a cardiovascular event, a new card was sent to the general practice.

4.7.4 Annual Questionnaire

Each participant also received an annual questionnaire enquiring about the development a heart attack, stroke, chest pain, leg pain, angina or hardening of the arteries during the last year. A cover letter reminding them about participation in the study and a prepaid envelop for returning the questionnaire was sent. Details of hospital attendances and aspirin medication were also requested. An example of the cover letter and questionnaire is shown on Appendix II. If a positive response on leg pain or chest pain was received from the annual questionnaire, a WHO IC or angina questionnaire was sent to the participant. The WHO IC and angina questionnaires are shown on Appendix I. If a positive response for other events was received, the general practice was contacted and the hospital records were searched to determine whether the event criteria were fulfilled.

4.7.5 Retrieval of hospital records

Participants who had a cardiovascular event during follow up identified from at least one of the following sources: the ISD computer print-out, the general practitioner or the annual questionnaires were further investigated by searching the hospital records. The records were first searched in hospitals cited in the ISD handouts, the general practitioner responses or the patient questionnaire. Permission to visit each hospital was requested by a letter signed by the study director. After September 2003, an event was confirmed if it appeared in the ISD handout and no other visits to the hospitals were performed.

4.8 Criteria for diagnosis of cardiovascular events

Criteria to define these events were adapted from the American Heart Association as described below (Gillum et al 1984; Hooi et al 2001). An event was recorded if these criteria were fulfilled.

4.8.1 Myocardial Infarction

1) Non-fatal MI

a) Definite MI was coded if two of the following criteria were present:

- i) Prolonged cardiac pain, anywhere in the anterior chest, left arm or jaw (possibly also involving back, shoulder, right arm or abdomen and lasting at least 20 min)
- ii) Diagnostic ECG codes, including Minnesota Codes: 1.1.1-1.2.5; 1.2.7; or 9.2 plus 5.1 or 5.2.
- iii) Elevated enzyme levels: creatine phosphokinase greater than twice the upper limits of normal, and one of the following also greater than twice the upper limits of normal: lactate dehydrogenase, glutamic oxalo-acetic transaminase, or MB isoenzyme of creatine phosphokinase. The enzymes must have been measured within 72 hours of an acute event.

b) Possible MI was coded if:

- i) One of the above defines criteria was present plus either
 - equivocal ECG codes: 1.2.8-1.3.6; 4.1-4.3; 5.1-5.3; or 9.2.

- equivocal enzyme levels: above normal but not twice normal or one was above twice normal but could be attributed to other cause.

ii) Equivocal ECG codes and equivocal enzyme levels

c) Silent MI was coded if:

i) ECG codes were diagnostic but elevated enzyme levels or cardiac pain was absent

2) Fatal MI

a) Definite MI was reported if one of the following criteria was present:

i) Post mortem evidence of acute MI

ii) Definite criteria for MI were present during the last 4 weeks prior to death

iii) International Classification of Diseases-9 (ICD) codes for cause of death were 410-414 or ICD-10 codes I21, I22, I24-I25.8 plus participant had a history of definite or possible MI; or plus definite or possible criteria for MI immediately preceding death; or plus post mortem evidence of severe coronary atherosclerosis or MI

b) Possible MI was coded if:

i) Death certificate codes were ICD-9 codes: 410-414 or ICD-10 codes: I21, I22, I24-I25.8 but no other evidence was found

4.8.2 Angina Pectoris

A diagnosis of angina during the follow up period required that there was no WHO evidence of angina at baseline plus either:

- i) Evidence of angina on the WHO questionnaire and recall of a doctor's diagnosis of angina
- ii) WHO angina plus ECG ischaemia
- iii) Clinical diagnosis of angina investigated by the general practitioner or in hospital

4.8.3 Stroke

- 1) Non-fatal stroke
 - a) Definite stroke was coded if one of the following criteria was present:
 - i) A history of onset of symptoms of less than 48 hours, plus clinical confirmation of a focal or global disturbance of cerebral function lasting more than 24 hours
 - ii) Computerised tomography scan showed evidence of cerebral infarction of haemorrhage
 - b) Possible stroke was coded if:
 - i) Primary or secondary discharge diagnosis included ICD-9 codes 431- 437 or ICD-10 codes I61-I67.9
- 2) Fatal stroke
 - a) Definite stroke was coded if one of the following was present:
 - i) Post mortem evidence of cerebral infarction or haemorrhage
 - ii) Criteria for definite stroke were met within 6 weeks prior to death
 - b) Possible stroke was coded if:

- i) Death certificate codes for underlying cause of death were ICD-9 431-437 or ICD-10 codes I61-I67.9

4.8.4 Transient Ischaemic Attack

A possible transient ischaemic attack was defined as a recorded history of rapid onset of clinical signs of focal or global disturbance of cerebral function lasting less than 24 hours.

4.8.5 Intermittent Claudication

Intermittent claudication was diagnosed using the WHO IC questionnaire.

4.8.6 Thrombo-embolism

Thrombo-embolism was coded if the diagnosis was confirmed by laboratory, radiological or surgical evidence.

4.8.7 Amputation/Surgery/ Angioplasty

A code of amputation referred to the amputation of any part of the lower limb, due to diabetes or vascular causes only. Vascular surgery, angioplasty, rest pain/ ulcer/ gangrene and CABG could be coded if these events were noted in participants' records.


4.9 Data analysis


This part describes the statistical analysis. Statistical tests and data handling are described for each section of the results separately. Analysis of the Edinburgh Artery Study data has been performed using SPSS v12-14. The information from

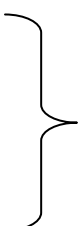
questionnaires and records forms was entered in a DBASE IV database from which the SPSS files were exported.

4.9.1 List of variables

The following variables were used in the subsequent analyses:

Cardiovascular risk factors and potential confounders:	
Age	 Measured at baseline
Sex	
Total cholesterol	
HDL cholesterol	
Total/ HDL cholesterol	
Smoking status	
Pack years of smoking	
BMI	
Diabetes	
Physical activity	
Hypertension	
History of CVD at baseline	

Inflammatory markers:	
CRP	 Measured at baseline
IL-6	
ICAM-1	
VCAM-1	
E-selectin	

Haemostatic markers:	
Fibrinogen	 Measured at baseline
D-dimer	
t-PA	
vWF	
Factor VII	
FpA	
F1+2	

Outcomes:	
Progression of peripheral atherosclerosis assessed by ABI change after 5 years and after 12 years	} After 17 years of follow up
Incident PAD	
Incident major CVD	
Incident MI	
Incident stroke	

4.9.2 Calculation and coding of variables

The following variables were calculated using the formulas shown below:

BMI= weight (kgr) / height² (m²)

Pack years of smoking: number of years smoked * average number of packs per day (zero was entered for lifelong non-smokers)

Total/ HDL cholesterol ratio: Total cholesterol/ HDL cholesterol

ABI: minimum [(left ankle systolic pressure/ left brachial systolic pressure), (right ankle systolic pressure/ right brachial systolic pressure)]

ABI change 5 years= ABI at 5 years- ABI at baseline

ABI change 12 years= ABI at 12 years- ABI at baseline

Also, the following categorical variables were coded as described below:

Smoking status was coded as a 2 level categorical variable. Subjects were classified as never smokers if they have answered to the questionnaire that they were never smokers and as ever smokers if they have answered that they were current or ex-smokers.

Physical activity was measured by a leisure activity questionnaire. A 4-group categorical variable was then calculated (no activity, light activity, moderate activity, and strenuous activity).

Diabetes status of subjects was defined by a number of ways. Subjects were classified as diabetic if they had answered the self-administrative questionnaire that they had been told by a doctor that they suffered from diabetes and were receiving either insulin or oral therapy. Also, a participant was recorded as suffering from diabetes if because of a doctor's diagnosis of diabetes they did not undergo the oral glucose intolerance test at baseline. Finally, if plasma glucose concentration was $\geq 7.8\text{mmol/L}$ or the 2h blood sample was $\geq 11.1\text{mmol/L}$ the subject was classified as diabetic.

Hypertension was defined as systolic blood pressure $\geq 140\text{mmHg}$ or diastolic blood pressure $\geq 90\text{mmHg}$ or when subject was self reported as taking drugs to lower his/her blood pressure.

History of CVD at baseline was defined as MI (2 out of the 3 of recall of doctor's diagnosis, WHO questionnaire and ECG ischemia), stroke (recall of doctor's diagnosis), angina (WHO questionnaire and either ECG ischemia or recall of doctor's diagnosis) or IC (WHO questionnaire).

Survival time was calculated in years by using the follow up of each participant from the baseline examination until death, most recent follow up assessment or until the event under examination (PAD, major CVD, MI, and stroke). Details for the calculation of each survival time are presented in the following sections.

Incident PAD was defined as an event of IC, rest pain, ulceration, amputation or vascular surgery during the follow up period. Criteria for IC were as defined in the previous section.

Incident major CVD was defined as fatal or non fatal MI, fatal or non fatal stroke,

CABG or angioplasty during the follow up period. Criteria for MI and stroke were as defined in the previous section.

Incident MI was defined as fatal or non fatal MI during the follow up period.

Incident stroke was defined as fatal or non fatal stroke during the follow up period.

4.9.3 Descriptive and cross-sectional analysis

The distributions of each cardiovascular risk factor, inflammatory marker and haemostatic marker were examined. Any extreme values/ outliers were investigated with the view of omitting from subsequent analysis. Any variable showing a skewed distribution was normalised using square root or log transformation as stated in the text. For skewed variables the median (IQR) is quoted in the text whereas for other continuous variables mean (SD) was used for descriptive statistics.

A correlation analysis using Pearson correlation was performed on cardiovascular risk factors, inflammatory and haemostatic markers in order to examine any associations between these variables, with variables having a skewed distribution being transformed appropriately.

The independent sample t-test was used to test differences between continuous cardiovascular risk factors, inflammatory and haemostatic markers and dichotomous variables. The chi squared test was used to test associations between categorical variables.

Throughout the following analysis multivariable models were performed with adjustments for potential confounders. Conventional cardiovascular risk factors were selected including age, sex, smoking, cholesterol levels, BMI, hypertension, diabetes, physical activity levels. As it was described in the introductory chapters these variables were interrelated with inflammatory and haemostatic markers and with CVD.

4.9.4 Progressive peripheral atherosclerosis

Progression of peripheral atherosclerosis was defined as the ABI change from baseline to 5 and to 12 years after follow up. The statistical significance of the change between ABI at 5 years and ABI at baseline or ABI at 12 years and ABI at baseline was tested with the t-test for paired samples.

Analysis of covariance (ANCOVA) was used to examine associations between cardiovascular risk factors, inflammatory and haemostatic markers and ABI at baseline, 5 years and 12 years. The partial correlation coefficients were used to test associations between cardiovascular risk factors, inflammatory and haemostatic markers and ABI at 5 years and 12 years adjusted for baseline ABI.

Linear regression was used to test associations between each cardiovascular risk factor and ABI change at 5 and 12 years. Analyses were initially adjusted for baseline ABI and further adjusted for all available cardiovascular risk factors risk factors: age, sex, pack years of smoking, presence of diabetes, total/ HDL cholesterol ratio, BMI, physical activity and history of CVD at baseline. Also, linear regression

was used to test associations between each inflammatory or haemostatic marker and ABI change at 5 and at 12 years. Analyses were initially adjusted for baseline ABI and further adjusted for risk factors: age, sex, pack years of smoking, presence of diabetes, total/ HDL cholesterol ratio, BMI, physical activity and history of CVD at baseline.

Different models with combinations of CRP, IL-6, fibrinogen and D-dimer as independent variables were investigated in order to find the model with the highest R^2 . Moreover, all inflammatory or all haemostatic markers were entered simultaneously into the linear regression model. Finally, the baseline population was divided in subgroups according to baseline history of CVD, total/ HDL cholesterol levels (above or below the median) and smoking status and linear regression was performed in each subgroup separately. In order to compare the effects of each marker on ABI change, the regression coefficients were standardised to represent the differences in ABI decline between the top and bottom tertiles of each marker. This was done by multiplying the regression coefficients by the inter-tertile range of the transformed distribution.

Odds ratios for transition from normal ($ABI > 0.9$) to low ABI ($ABI \leq 0.9$) were calculated using logistic regression adjusted for age sex and further adjusted for cardiovascular risk factors. In this analysis only subjects with normal ABI ($ABI > 0.9$) at baseline were included.

Also, subjects were divided into four mutually exclusive groups depending on their baseline levels of D-dimer and IL-6: subjects with D-dimer and IL-6 levels in the top tertiles, subjects with D-dimer and not IL-6 levels in the top tertile, subjects with IL-6 and not D-dimer levels in the top tertile and finally subjects with neither IL-6 nor D-dimer levels in the top tertiles. Similarly subjects were divided into four mutually exclusive groups depending on their baseline levels of D-dimer and CRP: subjects with D-dimer and CRP levels in the top tertiles, subjects with D-dimer and not IL-6 levels in the top tertile, subjects with CRP and not D-dimer levels in the top tertile and finally subjects with neither CRP nor D-dimer levels in the top tertiles. The trend between these groups and ABI change was tested for statistical significance using analysis of covariance adjusted for baseline ABI and for risk factors.

The interaction between age, sex, and levels of haemostatic or inflammatory markers was tested but the interaction term was not significant in any analysis. For the analysis of ABI change, linear regression models were adjusted for baseline ABI. Regression models without such adjustment were also performed. In these models, the estimates of the regression coefficients were essentially unchanged but the confidence intervals were wider and therefore data are not presented. Also, analyses were not adjusted for systolic blood pressure or for hypertension due to the high correlation between brachial blood pressure and the ABI.

Finally, mixed model analysis for repeated measures of ABI (baseline, 5 years and 12 years) was used to confirm results obtained from the linear regression analysis.

For this analysis, only subjects with ABI measurements at all 3 time points were included. Different covariance structure models were tested. Inflammatory and haemostatic markers were fitted as categorical (tertiles) in the analysis and the interaction term between inflammatory or haemostatic markers and time (baseline, 5 years, 12 years) was investigated.

4.9.5 Incident PAD

Only subjects without baseline IC were included in incident PAD analysis. To compare mean levels of risk factors at baseline in subjects who developed PAD and who did not, the independent sample t-test was used for continuous variables (after transformations where appropriate) and the chi-square test for categorical variables. A test for trend across disease categories and baseline risk factors was performed using analysis of variance.

Duration of follow up was calculated in years by using the follow up of each participant from the baseline examination until death, PAD event or most recent follow up assessment. Cox proportional hazard models were used to estimate risk of PAD. If more than one event was recorded (for example both IC and CLI), the first event was used for the calculation of the disease free survival time. Hazard ratios (95% CI) for BMI, diabetes, total/ HDL cholesterol ratio, hypertension, pack years of smoking, physical activity and CVD (MI, stroke or angina) at baseline adjusted for age and sex and then for all variables were calculated with Cox regression.

All inflammatory and haemostatic markers were fitted as categorical in the model (tertiles: three approximately equal groups) according to their baseline levels due to evidence for non-linearity between some markers levels and incident PAD (e.g. CRP levels and incident PAD). However, when alternative models fitting markers as continuous were performed results were essentially unchanged. Tertiles are also presented here for ease of interpretation and comparability with published meta-analysis on these markers and CHD. The hazard ratio for PAD of each inflammatory and haemostatic variable adjusted for age and sex (1) plus BMI, diabetes, total/HDL cholesterol ratio, pack years smoking, physical activity and hypertension (2) history of CVD at baseline (MI, stroke or angina) was calculated.

Cox regression with backward selection using inflammatory and haemostatic markers that were significant in univariable analysis was also performed. In this analysis cardiovascular risk factors and baseline CVD were forced to enter into the model.

Subsequent Cox regression models were fitted adjusting for all inflammatory markers, all haemostatic markers and combinations of those. Also, the baseline population was divided into six groups according (1) to baseline levels of total/ HDL ratio cholesterol (below or above median) and fibrinogen (top, middle or bottom tertile) (b) never or ever (present and ex) smokers and fibrinogen (top, middle or bottom tertile). Hazard ratios (95% CI) were calculated in each subgroup using the group of people with total/ HDL cholesterol below the median and fibrinogen at the

bottom tertile or the group of people who were never smokers and had fibrinogen levels at the bottom tertile as the reference group.

As in previous analysis, the interaction between age, sex, and levels of haemostatic or inflammatory markers was tested but the interaction term was not significant in any analysis.

4.9.6 Incident major CVD

The independent sample t-test was used to compare mean levels of risk factors at baseline in subjects who developed a cardiovascular event to those who did not, after data has been transformed as appropriate. Similarly, mean levels of all risk factors in subjects who developed MI or who developed stroke were compared to the group that remained healthy.

Duration of follow up was calculated in years by using the follow up of each participant from the baseline examination until death, CVD event or most recent follow up assessment. Cox proportional hazard models were used to estimate risk of combined major CVD, risk of MI and risk of stroke. Multiple events such as two MIs were counted only once. For the major CVD group, if both an MI and a stroke occurred, the first of the two events was used for the calculation of the disease free survival time.

Hazard ratios (95% CI) for BMI, diabetes, total/ HDL cholesterol ratio, hypertension, pack years of smoking, physical activity and baseline CVD (MI, stroke or angina) at baseline adjusted for age and sex and then for all variables were calculated with Cox regression.

Inflammatory and haemostatic variables were divided into tertiles (three approximately equal groups) according to their baseline levels. Hazard ratios (95% CI) between top and bottom tertiles of each marker were calculated and were subsequently adjusted for age and sex; (1) plus BMI, diabetes, total/HDL cholesterol ratio, pack years smoking, physical activity, history of CVD disease; and (2) plus ABI. Results from fitting markers as continuous were essentially unchanged and therefore tertiles are presented here for ease of interpretation and comparability with published meta-analysis. Subsequent Cox regression models were performed adjusting for all inflammatory markers, all haemostatic markers and combinations of those.

Cox regression with backward selection using inflammatory and haemostatic markers that were significant in univariable analysis was also performed. In this analysis cardiovascular risk factors, baseline CVD and the ABI were forced to enter into the model. Also, a composite score ranging from 0 to 3 for elevated concentrations of IL-6, fibrinogen and t-PA was calculated. One point for a value in the top tertile of each of the three variables was assigned. Cox regression was used to calculate hazard ratios (95%) for incident CVD for one, two or three elevated markers compared to none elevated. In addition, the baseline population was divided

into six groups according (1) to baseline levels of total/ HDL ratio cholesterol (below or above median) and CRP, IL-6, fibrinogen or D-dimer (top, middle or bottom tertile) (2) never or ever (present and ex) smokers and CRP, IL-6, fibrinogen or D-dimer (top, middle or bottom tertile). Hazard ratios (95% CI) were calculated in each subgroup using the group of people with total/ HDL cholesterol below the median and marker at the bottom tertile or the group of people who were never smokers and had CRP, IL-6, fibrinogen or D-dimer levels at the bottom tertile as the reference group.

This analysis was not adjusted for systolic blood pressure or hypertension due to their high correlation with the ABI. However, separate models adjusting for systolic blood pressure or hypertension and cardiovascular risk factors but not ABI were performed. Results for these models were not meaningfully different and data was not presented.

Finally, the interaction between age, sex, and levels of haemostatic or inflammatory markers was tested but the interaction term was not significant in any analysis.

4.9.7 Model assumptions and statistical considerations

Statistical analysis was performed using a predefined plan according to specific hypotheses that were to be tested each time. For every statistical test used, model assumptions were assessed and were found satisfactory unless otherwise stated in the text.

More specifically, normality of distributions was tested visually by plotting a histogram. Variables with skewed distributions were logarithmically or square root transformed as stated in the text. For the analysis of variance, the homogeneity of variance assumption was tested using the Levene's test. For linear and logistic regression analysis, tests were made for collinearity by scanning the correlation matrix between dependent variables and by looking at the variance inflation factor. Also standardised residuals were examined and only 1% of cases were expected to have standardised residuals outside ± 3 . Residual plots were investigated to look for heteroscedasticity. For Cox regression, the proportional hazards assumption was visually assessed by plotting the log-log survival curves and found to be satisfactory for all models constructed. A two sided p value < 0.05 was taken to denote statistical significance throughout all analyses.

Chapter five

5 Results: The study sample and associations of inflammatory and haemostatic markers with progressive peripheral atherosclerosis

5.1 Introduction

This chapter describes the population of the Edinburgh Artery Study at baseline and the interrelationships of inflammatory and haemostatic markers and cardiovascular risk factors. Associations between inflammatory and haemostatic markers and CVD at baseline (cross-sectionally) are also presented here.

In addition, in this chapter, the associations of inflammatory and haemostatic markers with peripheral progressive atherosclerosis after 5 and 12 years are presented. Progression of peripheral atherosclerosis was defined by the change in ABI after 5 and after 12 years. Each inflammatory and haemostatic marker has been associated with the progression of atherosclerosis in univariable analyses and in analyses adjusted for conventional cardiovascular risk factors. Models with all inflammatory and all haemostatic markers were also examined. The additive effect of these markers was tested and combinations of variables in relation to progressive atherosclerosis were investigated. Finally, their interaction with baseline cardiovascular risk factor levels was also tested.

Tables and figures are presented at the end of each section.

5.2 The study sample

This analysis describes the baseline population of the Edinburgh Artery Study and the characteristics of the subjects that participated in the 5 and 12 years clinical examination.

5.2.1 Age, sex and social class at baseline

At baseline 1,592 subjects were recruited. The mean age of the baseline population was 64.8 years and 51% of subjects were males. Table 13 shows that there was a slight under representation of subjects over 70 years old and of males aged 50-59 years. The social class distribution was similar to that of Edinburgh population from the 1981 census and 10.5% of the baseline population were classified in social class I, 31.7% in class II, 44.3% in class III, 9.3% in class IV and 3.5 in class V (Table 14).

5.2.2 Cardiovascular risk factors and prevalence of CVD at baseline

Conventional cardiovascular risk factors measured at baseline including BMI, diabetes status, smoking, cholesterol levels, blood pressure and physical activity are shown in Table 15. Six percent of the population suffered from diabetes mellitus and 26% were self-reported current cigarette smokers and 36% were ex-smokers. Also, 19% of the baseline population was receiving drugs to lower blood pressure and 57% were suffering from hypertension (defined as systolic blood pressure >120mmHg or diastolic blood pressure >90mmHg or use of anti-hypertensive drugs). Information on the use of other drugs such as aspirin or a statin was not available.

Also, 49 subjects (3 %) had suffered from stroke, 73 (4.6 %) had been diagnosed with MI and 158 (9.9 %) with angina prior to the baseline examination (Table 15). Finally, 73 (4.6 %) subjects were suffering from IC as identified by the WHO IC questionnaire. Of those with claudication, 32 (43.8%) had a grade 1 (severe pain), 24 (32.9) had grade 2 (mild pain) and 17 (23.3) had grade 3 (calf pain but not fulfilling every WHO criterion).

5.2.3 The study sample at 5 and 12 years follow up clinical examination

From the 1,592 subjects who were recruited at baseline; 1,156 participated in the 5 year follow up examination (131 additional subjects completed the questionnaire only); and 831 participated in the 12 year follow up (88 others completed the questionnaire only). Up to 5 years follow up, 203 deaths (12.8%) had occurred, making the total number of subjects with almost complete follow up data 1,490 (93.6%). Of the remaining 102 subjects: 18 did not wish to take part; 14 were unable to attend due to illness or hospitalization; 7 subjects were given appointments but failed to attend; 9 letters were returned by the Post Office; and 54 failed to answer the correspondence. By the 12 year examination, 485 deaths had been recorded (30.5%). Of the remaining 270 subjects, 105 were unwilling to take part in the examination, 21 have moved and could not be contacted, 74 have not been able to take part due to illness or frailty and 69 did not reply to the invitation.

Table 16 presents baseline characteristics for the baseline population and for the participants of each examination. There were not substantial differences between

these two groups; this shows that the people who attended the clinical examinations were broadly representative of the baseline population.

5.2.4 The study sample after 17 years of follow up

The baseline sample has been followed up for a mean 17 years for cardiovascular event. During this period 689 subjects have died (43% of the baseline population). Of those, 281 (41%) subjects have died due to CVD. In particular, 129 (19%) deaths were due to MI, 65 (9%) due to stroke and 83 (12%) due to other cardiovascular events. Survival curves for all cause and cardiovascular mortality are shown in Figure 5.

During the follow up, 648 subjects had at least one cardiovascular event recorded including MI, stroke, angina, IC, and other cardiovascular events. In detail, 147 non fatal events of MI and 106 of stroke were recorded whereas 209 subjects developed symptomatic PAD during the subsequent 17 years defined as IC, rest pain, gangrene ulcer, amputation or vascular surgery.

Table 13 Age by sex distribution of the baseline population

Age (years)	Males (%)	Females (%)
55-59	182 (22.5)	216 (27.6)
60-64	205 (25.3)	201 (25.7)
65-69	228 (28.2)	199 (25.4)
70-74	194 (24.0)	167 (21.3)
Total	809 (100.0)	783 (100.0)

Table 14 Social class of study members and residents of Edinburgh city

Social class	Attendees (55-74 years) (n=1,592)	Edinburgh city [†] (>16 years) (n=31,382)
I	10.5 %	10.0 %
II	31.5 %	25.0 %
IIIN	16.0 %	16.0 %
IIIM	28.0 %	28.0 %
IV	9.3 %	13.0 %
V	3.5 %	6.0 %
Unclassified	1.2 %	2.0 %

[†] Based on 10% sample of the 1981 census

Table 15 Baseline population characteristics

	N	Mean (SD)/ Percent
Age (years)	1,592	64.8 (5.67)
Males (%)	809	50.9
BMI (kg/m ²)	1,591	25.6 (3.91)
Total cholesterol (mmol/L)	1,573	7.03 (1.33)
HDL cholesterol (mmol/L)	1,567	1.44 (0.41)
Total/ HDL cholesterol ratio	1,567	5.24 (1.96)
Pack years smoking (among smokers) [†]	1,592	23.9 (4.12)
Diabetes (%)	91	6.0
Blood Pressure		
Systolic Blood Pressure (mmHg)	1,591	144.5 (24.12)
Diastolic Blood Pressure (mmHg)	1,587	77.4 (12.42)
Antihypertensives [‡] (%)	307	19.2
Cardiovascular disease (%)		
Myocardial infarction	73	4.6
Stroke	49	3.1
Angina	107	6.7
Intermittent claudication	73	4.6
Physical activity (%)		
Light	592	37.2
Moderate	674	42.3
Strenuous	167	10.5

[†] Pack years have been calculated from square root transformed variable and have been back transformed here

[‡] People receiving drugs to lower blood pressure

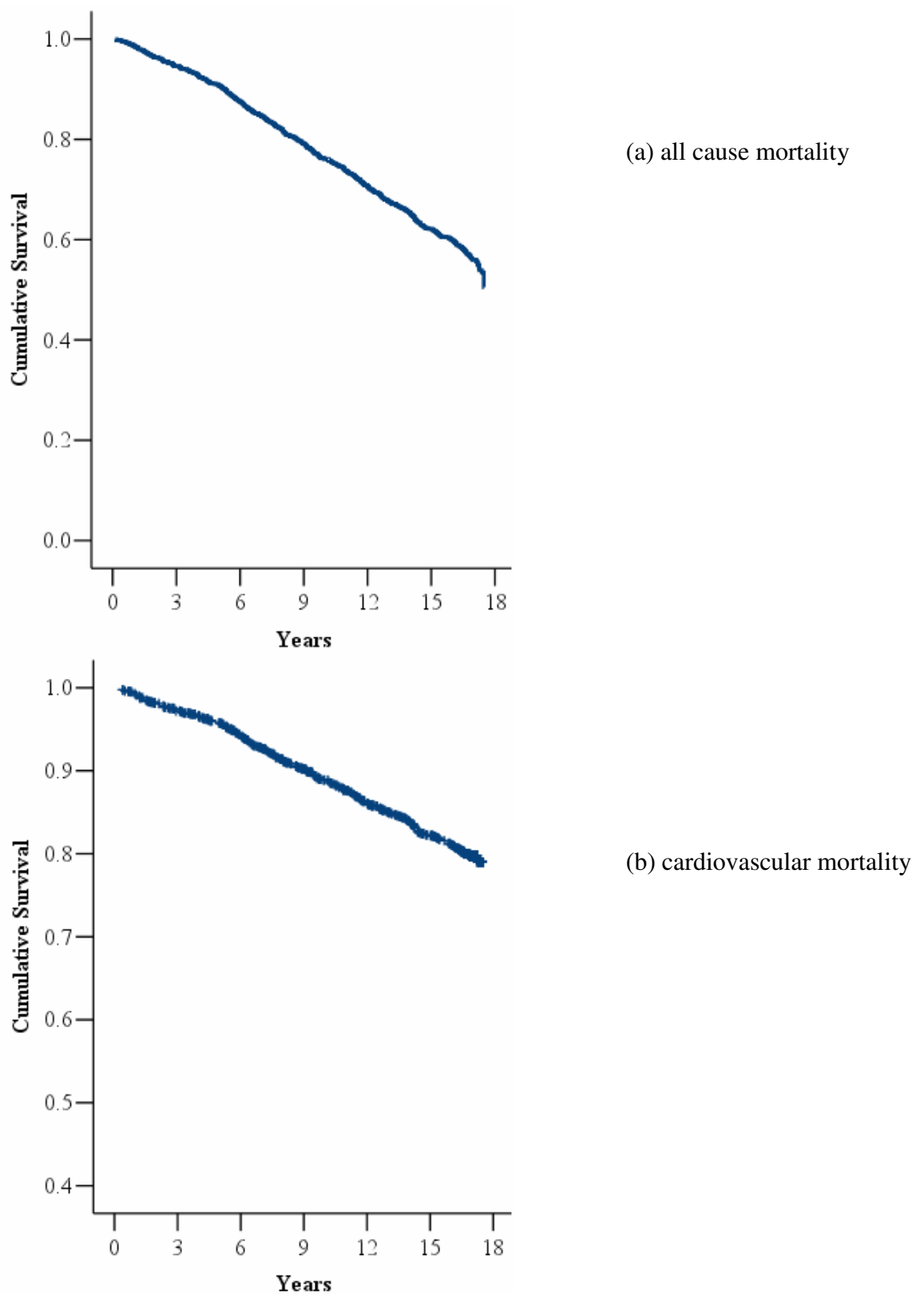
Table 16 Population characteristics at baseline of all subjects and of those who participated at each examination

	Mean (SD)/ Percent		
	Whole population (N=1,592)	Attenders 5 year (N=1,156)	Attenders 12 year (N=831)
Age (years)	64.8 (5.7)	64.2 (5.6)	63.2 (5.3)
Males (%)	50.9	49.8	48.3
BMI (kg/m ²)	25.6 (3.91)	25.6 (3.90)	25.5 (3.84)
Total cholesterol (mmol/L)	7.03 (1.33)	7.06 (1.32)	7.06 (1.59)
HDL cholesterol (mmol/L)	1.44 (0.41)	1.45 (0.39)	1.46 (0.39)
Total/ HDL cholesterol ratio	5.24 (1.96)	5.18 (1.67)	5.12 (1.50)
Pack years smoking [†]	23.9 (4.12)	22.0 (3.65)	20.43 (3.50)
Diabetes (%)	6.0	4.4	3.6
Blood Pressure (mmHg)			
Systolic Blood Pressure	144.5 (24.12)	142.6 (23.24)	140.1 (22.59)
Diastolic Blood Pressure	77.4 (12.42)	77.1 (12.14)	76.7 (11.87)
Antihypertensives [‡] (%)	19.2	12.2	10.6
CVD (%)			
Myocardial Infarction	4.6	2.9	2.3
Stroke	3.1	2.2	1.1
Angina	6.7	7.5	6.1
Intermittent Claudication	4.6	3.8	2.6

[†] Pack years have been calculated from square root transformed variable and have been back transformed here

[‡] People receiving drugs to lower blood pressure

Figure 5 Survival curve for (a) all cause and (b) cardiovascular mortality over 17 years of follow up



5.3 Inflammatory and haemostatic markers at baseline

This analysis focuses on the distribution and the interrelationships of inflammatory and haemostatic markers measured at baseline. Also, it describes their associations with baseline cardiovascular risk factors and CVD.

5.3.1 Distribution of inflammatory and haemostatic markers

Histograms of all markers are shown in Figure 6. Fibrinogen had a frequency distribution close to normal. Distributions of t-PA and factor VII were positively skewed and were square root transformed in subsequent analyses that assume approximate normality. Distributions of IL-6, ICAM-1, VCAM-1, E-selectin, D-dimer, vWF, F1+2 and FpA displayed higher skewness and were logarithmically transformed. Also, 98 subjects with CRP levels above 10mg/l and 11 subjects with IL-6 levels above 100pg/ml were excluded from all analyses because these levels indicate presence of acute inflammatory disease.

Median levels (interquartile range (IQR)) of each inflammatory and haemostatic marker in the general population and for males and females separately are shown in Table 17. Levels of CRP, ICAM-1, VCAM-1 and vWF did not show any significant differences between males and females. On the contrary, significantly higher levels in males than females were observed for IL-6, E-selectin, t-PA and FpA ($p < 0.001$). Fibrinogen ($p < 0.001$), D-dimer ($p = 0.02$), factor VII ($p < 0.001$) and F1+2 ($p < 0.001$) were significantly higher in female subjects.

5.3.2 Correlations between inflammatory and haemostatic markers

Overall, markers studied here were highly correlated (Table 18). The highest correlations were observed between CRP, IL-6 and fibrinogen which all had correlation coefficients close or greater to 0.5 ($p < 0.001$). Haemostatic markers, factor VII, FpA and F1+2, did not show strong associations with other markers studied here and displayed several non-significant correlations coefficients.

5.3.3 Associations between inflammatory and haemostatic markers with cardiovascular risk factors

Table 19 shows the correlation coefficients between inflammatory and haemostatic markers and cardiovascular risk factors. CRP, IL-6, ICAM-1, fibrinogen, D-dimer and t-PA were strongly correlated with these factors whereas other markers displayed weak correlations. Negative correlations were observed between CRP, IL-6, ICAM-1, fibrinogen, D-dimer, and t-PA and levels of HDL cholesterol. On the contrary these markers were positively correlated with total cholesterol levels and with systolic blood pressure. Correlations between these markers and diastolic blood pressure were much weaker.

Smoking

Also, inflammatory markers CRP, IL-6 and ICAM-1 showed strong correlations with pack years of smoking ($r = 0.2, 0.3$ and 0.3 respectively) which were higher than those observed between haemostatic markers and smoking (for fibrinogen and D-dimer $r = 0.1$). When the population was divided into never and ever (present and

ex) smokers, CRP, IL-6, ICAM-1, fibrinogen, t-PA, vWF and FpA were significantly higher in ever smokers than in never smokers. Median (IQR) levels of each marker according to smoking status are shown in Table 20.

Diabetes

Levels of all inflammatory markers showed a significant increase in subjects with diabetes at baseline compared to those without (Table 21). From the haemostatic markers, t-PA and vWF showed a significant increase in subjects with diabetes. Fibrinogen had higher levels in subjects with diabetes (media (IQR) 2.73 (2.12, 3.32)) compared to those without (median (IQR) 2.63 (2.23, 3.10)) but this difference did not reach statistical significance. Similarly, levels of D-dimer, factor VII, F1+2 and FpA did not show any significant differences between subjects with and without diabetes at baseline.

Physical activity

Moreover, inflammatory markers, except E-selectin, were lower with increasing physical activity categories from ‘no activity’ to ‘light’, ‘moderate’ and ‘strenuous activity’ and showed a significant trend across physical activity categories as presented in Table 22. From the haemostatic markers, only fibrinogen and D-dimer had a significant trend across increasing physical activity status at baseline (Table 22).

Hypertension

Median levels (IQR) of all markers except factor VII and FpA were significantly higher in subjects with hypertension compared to subjects without hypertension and their median (IQR) levels are shown in Table 23.

5.3.4 Associations between inflammatory and haemostatic markers with CVD at baseline

In general, plasma biomarkers were elevated in people suffering from CVD at baseline. Table 24 lists median (interquartile range) in the group of people with MI and/ or angina and stroke compared to those who were healthy (no MI, angina, stroke or IC at baseline). Inflammatory markers CRP, IL-6, ICAM-1 and VCAM-1 ($p < 0.01$) were significantly elevated in the CHD group. These markers were also higher in subjects with stroke but only levels of IL-6 and VCAM-1 achieved statistical significance, probably due to the small sample size of the stroke group. For example median (IQR) of IL-6 2.03 (1.09, 2.97) in the group of people without baseline CVD but in those who had MI and/ or angina or stroke it was increased to 3.20 (1.13, 5.27) and 2.29 (0.45, 4.14) respectively.

Similarly, fibrinogen, D-dimer and t-PA were significantly higher in subjects suffering from MI and/ or angina or stroke compared to the group that were free of CVD at baseline ($p < 0.01$). vWF ($p < 0.01$) was significantly elevated in subjects with MI and/ or angina only. When the group of subjects with either MI or angina was divided to those with MI only or angina only, CRP, IL-6, VCAM-1, fibrinogen, D-dimer and vWF ($p < 0.01$) were significantly elevated in either the MI or the angina

group compared to the healthy group. Nevertheless, t-PA ($p < 0.01$) and ICAM-1 ($p < 0.05$) were significantly higher in the group suffering from angina only and their median (IQR) was 7.98 (5.56, 10.4) and 134 (99, 168) respectively.

Furthermore, in the 73 subjects suffering from IC at baseline, CRP ($p < 0.001$), IL-6 ($p < 0.001$), ICAM-1 ($p = 0.03$), VCAM-1 ($p = 0.04$), fibrinogen ($p < 0.001$), D-dimer ($p < 0.001$) and vWF ($p = 0.08$) were significantly elevated compared to the healthy group of no CVD disease at baseline (Table 24). When individuals were classified as worsening PAD: from normal, asymptomatic ($ABI \leq 0.9$) to symptomatic (WHO questionnaire) a significant trend was found between higher plasma levels of inflammatory markers: CRP ($p < 0.001$), IL-6 ($p < 0.001$), ICAM-1 ($p < 0.01$) and VCAM-1 ($p = 0.03$) (Table 25). In addition, haemostatic markers fibrinogen ($p < 0.001$), D-dimer ($p < 0.001$), t-PA ($p < 0.001$) and vWF ($p = 0.008$) displayed a significant trend across worsening PAD (Table 25).

Figure 6 Histograms of inflammatory and haemostatic variables measured at baseline

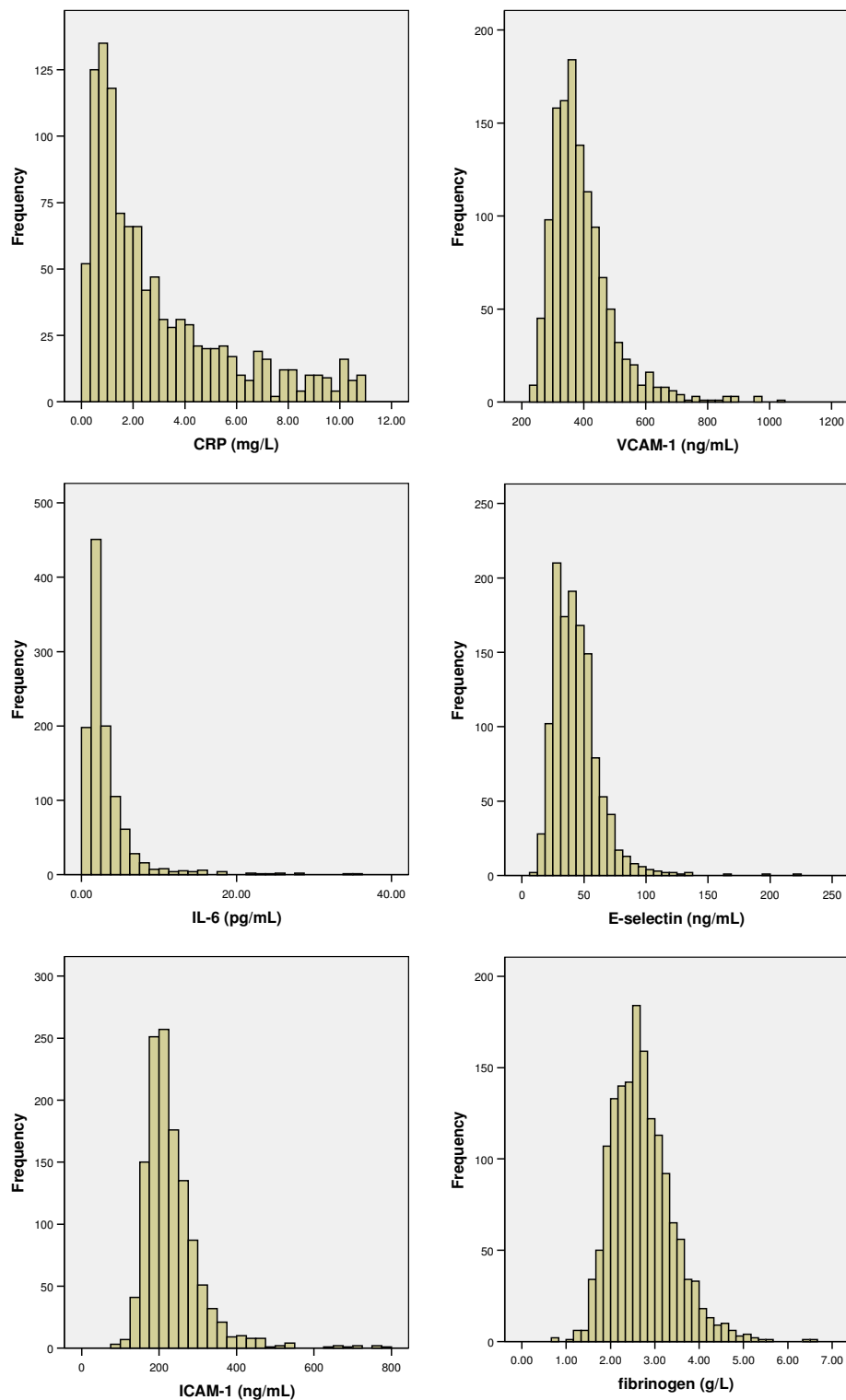
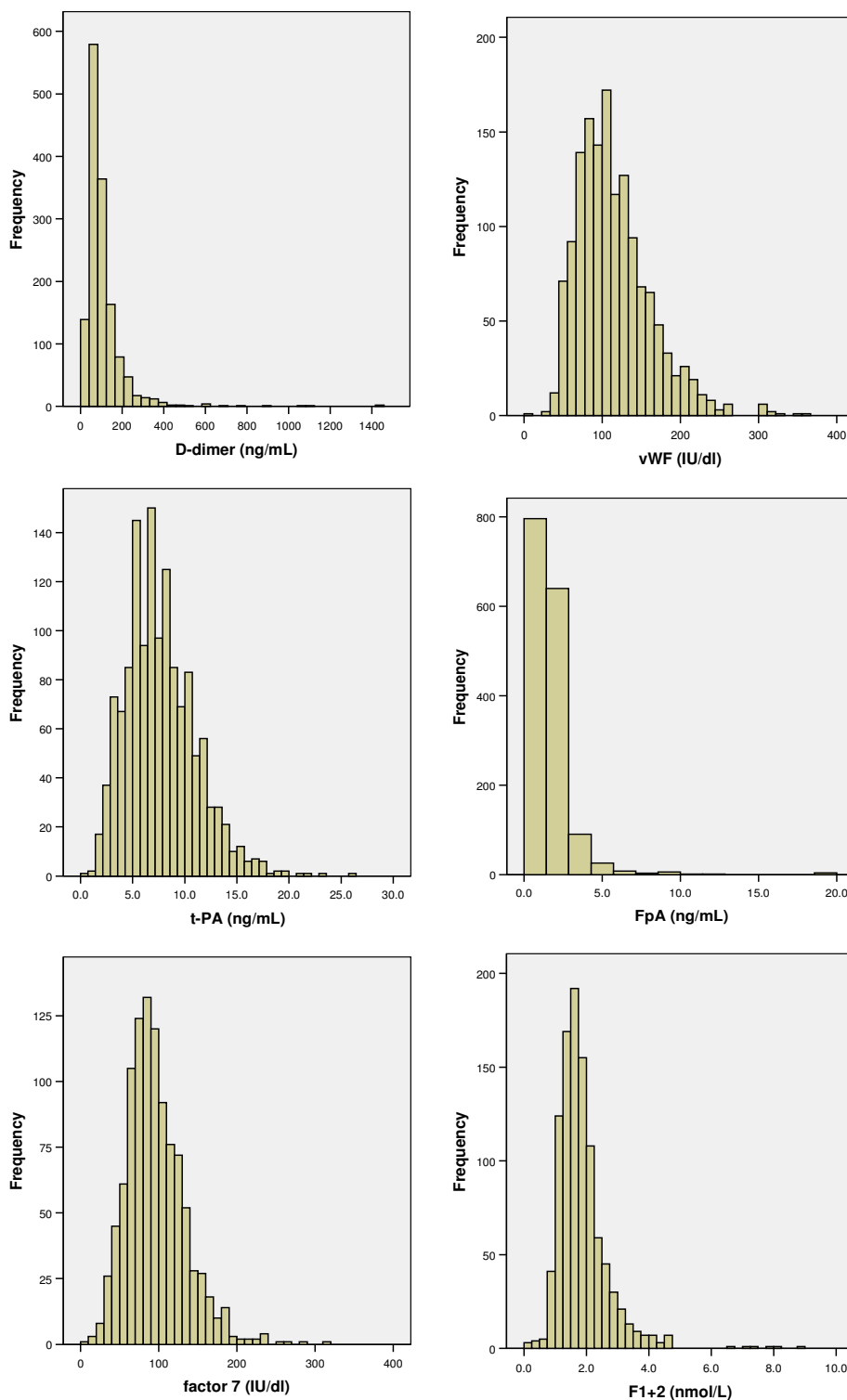


Figure 6 cont.



CRP: C-reactive protein, IL-6: interleukin-6, ICAM-1: intercellular adhesion molecule 1, VCAM-1 : vascular adhesion molecule 1, t-PA: tissue plasminogen activator 1, vWF : vonWillebrand factor, FpA : fibrinopeptide A, F 1+2 : prothrombin fragments 1+2

Table 17 Levels of inflammatory and haemostatic markers at baseline in males and females

	N	All subjects	Median (Interquartile range)		P value [†]
			Males	Females	
Inflammatory markers					
CRP (mg/L)	992	1.85 (0.87, 3.43)	1.86 (0.92, 4.07)	1.83 (0.89, 4.02)	0.74
IL-6 (pg/mL)	1,038	2.17 (1.08, 3.26)	2.41 (1.58, 3.99)	1.91 (1.31, 3.12)	<0.001
ICAM-1 (ng/mL)	1,182	216 (181, 251)	215 (187, 256)	218 (188, 261)	0.34
VCAM-1 (ng/mL)	1,182	371 (316, 425)	370 (325, 443)	371 (325, 427)	0.35
E-selectin (ng/mL)	1,179	41 (30, 51)	43 (32, 54)	39 (30, 50)	<0.001
Haemostatic markers					
Fibrinogen (g/L)	1,548	2.63 (2.19, 3.07)	2.55 (2.14, 3.03)	2.72 (2.33, 3.16)	<0.001
D-dimer (ng/mL)	1,436	83 (51, 116)	80 (56, 120)	88 (61, 131)	0.02
t-PA (ng/mL)	1,362	7.3 (5.0, 9.5)	8.0 (5.9, 10.3)	6.5 (4.8, 8.8)	<0.001
vWF (IU/dl)	1,446	107 (78, 136)	108 (82, 140)	105 (80, 139)	0.49
Factor VII (IU/dl)	1,031	91 (67, 158)	84 (65, 110)	95 (75, 122)	<0.001
F1+2 (ng/mL)	1,580	1.69 (1.56, 2.09)	1.62 (1.29, 2.05)	1.76 (1.42, 2.19)	0.001
FpA (nmol/L)	1,062	1.40 (1.35, 1.45)	1.50 (1.00, 2.00)	1.30 (1.00, 1.80)	0.001

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1,

t-PA: tissue plasminogen, vWF: von Willebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2

[†] p value for difference between males and females calculated with the t-test using transformed inflammatory and haemostatic markers.

Table 18 Pearson correlation coefficient matrix between transformed inflammatory and haemostatic markers

	CRP (mg/L)	IL-6 (pg/mL)	ICAM-1 (ng/mL)	VCAM-1 (ng/mL)	E-selectin (ng/mL)	Fibrinogen (g/L)	D-dimer (ng/mL)	t-PA (ng/mL)	vWF (IU/dl)	F1+2 (ng/mL)	Factor VII (IU/dl)
IL-6	0.51	1									
ICAM-1	0.29	0.30	1								
VCAM-1	0.10	0.21	0.38	1							
E-selectin	0.25	0.19	0.36	0.16	1						
Fibrinogen	0.51	0.48	0.26	0.09	0.09	1					
D-dimer	0.24	0.33	0.20	0.14	0.08	0.38	1				
t-PA	0.27	0.27	0.19	0.11	0.25	0.15	0.12	1			
vWF	0.18	0.23	0.22	0.31	-0.004	0.22	0.20	0.12	1		
F1+2	0.01	0.05	0.07	0.04	-0.02	-0.05	0.28	-0.10	-0.02	1	
Factor VII	0.09	0.005	0.08	0.02	0.04	0.12	0.03	0.09	0.10	0.06	1
FpA (nmol/L)	0.10	0.21	0.08	0.06	0.06	0.07	0.16	0.02	0.13	-0.01	-0.07

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1, t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2
Correlation coefficients in bold are statistically significant at 0.01 level

Table 19 Pearson correlation coefficients between transformed inflammatory and haemostatic markers and continuous cardiovascular risk factors

	Age (years)	BMI (kg/m ²)	Total cholesterol (mmol/L)	HDL cholesterol (mmol/L)	Total/ HDL cholesterol ratio	Pack years smoking	Systolic Blood Pressure (mmHg)	Diastolic Blood Pressure (mmHg)
CRP (mg/L)	0.15	0.29	0.004	-0.18	0.19	0.20	0.15	0.10
IL-6 (pg/mL)	0.20	0.13	-0.12	-0.23	0.14	0.27	0.13	0.07
ICAM-1 (ng/mL)	0.11	0.03	-0.03	-0.18	0.14	0.30	0.11	0.02
VCAM-1 (ng/mL)	0.22	0.02	-0.19	-0.17	0.04	0.01	0.18	0.08
E-selectin (ng/mL)	-0.05	0.20	-0.02	-0.15	0.13	0.06	0.05	0.11
Fibrinogen (g/L)	0.19	0.15	0.09	-0.12	0.14	0.15	0.12	0.05
D-dimer (ng/mL)	0.37	0.07	0.06	-0.06	0.09	0.08	0.24	0.11
t-PA (ng/mL)	0.17	0.25	0.03	-0.28	0.27	0.21	0.17	0.15
vWF (IU/dl)	0.23	0.02	-0.04	-0.04	0.01	0.10	0.12	-0.01
Factor VII (IU/dl)	0.06	0.11	0.19	0.04	0.06	-0.06	0.10	0.03
F1+2 (ng/mL)	0.17	-0.03	0.08	0.05	-0.02	0.005	0.10	0.03
FpA (nmol/L)	0.08	0.01	-0.06	-0.05	0.03	0.08	0.01	0.002

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1, t-PA: tissue plasminogen, vWF: von Willebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.
Correlation coefficients in bold are statistically significant at 0.01 level.

Table 20 Levels of inflammatory and haemostatic markers according to smoking status at baseline

	Ever Smokers	Never smokers	P value for difference [†]
	Median (Interquartile range)		
Inflammatory markers			
CRP (mg/L)	2.21 (1.02, 4.65)	1.40 (0.75, 2.91)	< 0.001
IL-6 (pg/mL)	2.35 (1.59, 3.96)	1.80 (1.20, 3.06)	< 0.001
ICAM-1 (ng/mL)	229 (195, 275)	203 (195, 275)	< 0.001
VCAM-1 (ng/mL)	368 (322, 433)	373 (330, 436)	0.65
E-selectin (ng/mL)	42 (31, 51)	40 (31, 51)	0.39
Haemostatic markers			
Fibrinogen (g/L)	2.71 (2.28, 3.20)	2.55 (2.13, 2.94)	< 0.001
D-dimer (ng/mL)	87 (59, 126)	80 (58, 116)	0.40
t-PA (ng/mL)	7.60 (5.60, 10.00)	6.50 (4.90, 9.00)	< 0.001
vWF (IU/dl)	109 (82, 143)	103 (78, 133)	0.01
Factor VII (IU/dl)	91 (69, 115)	90 (71, 119)	0.20
F1+2 (ng/mL)	1.73 (1.35, 2.14)	1.66 (1.32, 2.12)	0.58
FpA (nmol/L)	1.50 (1.00, 2.00)	1.30 (1.00, 1.80)	0.001

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1, t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

[†] p value between ever and never smokers calculated with the t-test using transformed inflammatory and haemostatic markers.

Table 21 Levels of inflammatory and haemostatic markers according to diabetes status at baseline

Baseline	Normal	Diabetes	P value for difference [†]
	Median (Interquartile range)		
Inflammatory markers			
CRP (mg/L)	1.80 (0.87, 3.89)	3.35 (1.35, 5.36)	0.005
IL-6 (pg/mL)	2.12 (1.41, 3.60)	2.61 (1.87, 4.00)	0.02
ICAM-1 (ng/mL)	215 (187, 256)	238 (209, 311)	< 0.001
VCAM-1 (ng/mL)	370 (324, 432)	395 (354, 451)	0.001
E-selectin (ng/mL)	41 (31, 51)	49 (36, 65)	< 0.001
Haemostatic markers			
Fibrinogen (g/L)	2.63 (2.23, 3.10)	2.73 (2.12, 2.32)	0.33
D-dimer (ng/mL)	83 (58, 123)	90 (65, 126)	0.25
t-PA (ng/mL)	7.10 (5.00, 9.50)	8.95 (7.00, 10.60)	< 0.001
vWF (IU/dl)	106 (80, 139)	127 (98, 158)	< 0.001
Factor VII (IU/dl)	90 (70, 115)	91 (67, 131)	0.57
F1+2 (ng/mL)	1.70 (1.33, 2.12)	1.64 (1.36, 2.24)	0.10
FpA (nmol/L)	1.4 (1.0, 2.0)	1.5 (1.0, 2.1)	0.80

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1, t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

[†] p values between subjects with and without diabetes calculated with the t-test using transformed inflammatory and haemostatic markers.

Table 22 Levels of inflammatory and haemostatic markers according to physical activity status at baseline

	No activity	Light activity	Moderate activity	Strenuous activity	P value for trend [†]
Median (Interquartile range)					
Inflammatory markers					
CRP (mg/L)	2.74 (1.65, 4.22)	2.16 (1.01, 4.37)	1.60 (0.75, 3.43)	2.13 (1.43, 3.22)	0.01
IL-6 (pg/mL)	2.58 (1.65, 4.22)	2.21 (1.46, 3.74)	2.02 (1.33, 3.41)	1.68 (0.84, 3.51)	0.004
ICAM-1 (ng/mL)	226 (197, 275)	226 (195, 270)	209 (183, 247)	211 (180, 245)	< 0.001
VCAM-1 (ng/mL)	385 (327, 449)	382 (331, 444)	360 (320, 420)	365 (327, 449)	0.03
E-selectin (ng/mL)	41 (30, 51)	41 (31, 53)	41 (31, 51)	43 (33, 53)	0.27
Haemostatic markers					
Fibrinogen (g/L)	2.67 (3.32, 3.33)	2.74 (2.33, 3.15)	2.57 (2.17, 3.03)	2.53 (2.12, 3.04)	0.01
D-dimer (ng/mL)	91 (62, 135)	91 (63, 134)	79 (56, 113)	76 (49, 119)	0.003
t-PA (ng/mL)	8.0 (5.0, 10.0)	7.4 (5.0, 9.5)	7.0 (5.2, 9.5)	7.3 (5.4, 10.0)	0.98
vWF (IU/dl)	116 (87, 148)	108 (81, 142)	105 (81, 137)	104 (78, 132)	0.15
Factor VII (IU/dl)	93 (73, 121)	95 (70, 122)	89 (69, 112)	87 (67, 104)	0.51
F1+2 (ng/mL)	1.65 (1.40, 2.17)	1.77 (1.36, 2.27)	1.66 (1.30, 2.01)	1.65 (1.29, 2.05)	0.29
FpA (nmol/L)	1.50 (1.00, 2.10)	1.30 (1.00, 1.80)	1.50 (1.00, 2.00)	1.50 (1.00, 2.20)	0.78

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1, t-PA: tissue plasminogen, vWF: von Willebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

[†] calculated with the chi-square using the transformed inflammatory and haemostatic markers.

Table 23 Levels of inflammatory and haemostatic markers according to hypertension status at baseline

Baseline	Hypertension [†]	Normal blood pressure	P value for difference [‡]
	Median (Interquartile range)		
Inflammatory markers			
CRP (mg/L)	2.16 (1.01, 3.97)	1.54 (0.76, 3.63)	< 0.001
IL-6 (pg/mL)	2.34 (1.53, 4.32)	1.93 (1.29, 3.18)	< 0.001
ICAM-1 (ng/mL)	223 (194, 265)	210 (182, 250)	0.002
VCAM-1 (ng/mL)	386 (335, 447)	356 (315, 413)	0.001
E-selectin (ng/mL)	42 (31, 53)	39 (30, 51)	0.05
Haemostatic markers			
Fibrinogen (g/L)	2.69 (2.28, 3.20)	2.58 (2.17, 2.94)	< 0.001
D-dimer (ng/mL)	92 (59, 126)	75 (51, 116)	< 0.001
t-PA (ng/mL)	7.80 (5.60, 10.00)	6.50 (4.70, 9.00)	< 0.001
vWF (IU/dl)	109 (83, 145)	104 (78, 133)	< 0.001
Factor VII (IU/dl)	92 (70, 119)	89 (70, 119)	0.10
F1+2 (ng/mL)	1.72 (1.35, 2.22)	1.66 (1.32, 2.12)	0.01
FpA (nmol/L)	1.40 (1.00, 2.00)	1.50 (1.00, 1.80)	0.90

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1, t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

[†] defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg or use of drugs to lower blood pressure.

[‡] between subjects with hypertension and normal blood pressure calculated with the t-test using transformed inflammatory and haemostatic markers.

Table 24 Levels of inflammatory and haemostatic markers according to cardiovascular disease status at baseline

	MI and/ or Angina (N= 191)	Stroke (N=49)	Healthy [†] (N=1,315)
Inflammatory markers			
CRP (mg/L)	3.20 (1.13, 5.27)**	2.29 (0.45, 4.14)	1.68 (0.27, 3.09)
IL-6 (pg/mL)	3.02 (1.52, 4.52)**	3.34 (2.38, 3.20)**	2.03 (1.09, 2.97)
ICAM-1 (ng/mL)	230.0 (188, 272)**	235.0 (195.5, 274.5)	214.0 (179, 249)
VCAM-1 (ng/mL)	389.0 (321, 457)**	404.0 (348.2, 459.7)*	368.0 (315.5, 420.5)
E-selectin (ng/mL)	42.0 (30.0, 50.0)	44.0 (32, 56)	41.0 (30.5, 51.5)
Haemostatic markers			
Fibrinogen (g/L)	2.88 (2.45, 3.31)**	2.89 (1.44, 3.49)**	2.60 (2.17, 3.03)
D-dimer (ng/mL)	101.5 (55.6, 157.1)**	103.5 (64.6, 142.4)**	80.0 (50.5, 109.5)
t-PA (ng/mL)	8.10 (5.86, 10.35)**	9.7 (8.6, 11.9)**	7.0 (5.85, 9.15)
vWF (IU/dl)	123.0 (92.2, 153.7)**	118.5 (94.6, 142.4)	104.0 (74.5, 133.5)
Factor VII (IU/dl)	91 (63, 119)	102.0 (61.5, 142.5)	90.0 (67.0, 123.0)
F1+2 (ng/mL)	1.75 (1.31, 2.18)	1.65 (1.28, 2.02)	1.68 (1.28, 2.08)
FpA (nmol/L)	1.50 (1.0, 2.0)	1.50 (0.9, 2.1)*	1.40 (1.35, 1.45)

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1, t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2, MI: myocardial infarction

[†] No MI, angina, stroke or IC at baseline.

** p value for difference between MI and/or angina or stroke group with the healthy group < 0.01, p values have been calculated using the transformed distributions of inflammatory and haemostatic markers.

* p value for difference between MI and/or angina or stroke group with the healthy group < 0.05 level, p values have been calculated using the transformed distributions of inflammatory and haemostatic markers.

Table 25 Levels of inflammatory and haemostatic markers according to baseline Peripheral Arterial Disease (PAD) status

Table 2: Levels of inflammatory and haemostatic markers according to subgroups of patients (n=100) Status						
	Symptomatic	Median (Interquartile range)		P value		P value for trend
		Asymptomatic	Normal	Symptomatic vs. normal	Asymptomatic vs. normal	
Inflammatory markers						
CRP (mg/L)	3.66 (1.72, 5.13)	2.61(1.04, 4.40)	1.57(0.76, 3.07)	<0.001	<0.001	<0.001
IL-6 (pg/mL)	2.96 (1.78, 5.03)	2.60 (1.63, 4.24)	2.01 (1.32, 3.17)	<0.001	<0.001	<0.001
ICAM-1 (ng/mL)	234 (204, 275)	232 (198, 275)	212 (183, 250)	0.01	<0.001	<0.001
VCAM-1 (ng/mL)	405 (330, 457)	386 (336, 444)	365 (322, 426)	0.03	0.03	0.03
E-selectin (ng/mL)	44 (32, 52)	43 (33, 53)	40 (30, 52)	0.68	0.02	0.17
Haemostatic markers						
Fibrinogen (g/L)	3.00 (2.47, 3.70)	2.76 (2.39, 3.24)	2.58 (2.16, 3.01)	<0.001	<0.001	<0.001
D-dimer (ng/mL)	112.0 (81.0, 185.0)	99.0 (68.0, 150.0)	78.0 (55.0, 110.0)	<0.001	<0.001	<0.001
t-PA (ng/mL)	7.70 (5.80, 9.80)	8.00 (5.50, 10.50)	7.00 (5.00, 9.10)	0.08	<0.001	<0.001
vWF (IU/dl)	118.0 (92.0, 158.0)	109.0 (82.0, 148.0)	104.0 (79.0, 137.0)	0.01	0.13	0.01
Factor VII (IU/dl)	88.5 (66.0, 112.0)	95.0 (72.0, 117.0)	88.5 (69.0, 115.0)	0.91	0.11	0.91
F1+2 (ng/mL)	1.40 (1.00, 2.00)	1.40 (1.00, 2.00)	1.40 (1.00, 2.00)	0.11	0.42	0.11
FpA (nmol/L)	1.72 (1.42, 2.29)	1.74 (1.38, 2.23)	1.68 (1.32, 2.10)	0.83	0.53	0.83

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1,

t-PA: tissue plasminogen, vWF: von Willebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

† Symptomatic: Intermittent Claudication (WHO questionnaire), Asymptomatic: ankle brachial index ≤ 0.9 , Normal: None of the above.

‡ P values have been calculated using the transformed distributions of inflammatory and haemostatic markers.

5.4 Peripheral atherosclerosis measured by the ABI

This analysis describes the distribution of ABI at three time points along the follow up period and the mean ABI change along these periods.

5.4.1 The ABI at three time points

The ABI was measured at the baseline, 5 years and 12 years follow up of the Edinburgh Artery Study. Valid ABI measurements were available for 1,582 subjects (99% of participants) at baseline, for 1,081 subjects (93.1%) at the 5 year follow up and for 816 subjects (98.2%) at the 12 year follow up. Three, 5 and 8 subjects at baseline, 5 years and 12 years, respectively, had values of ABI above 1.50 and were excluded from all analyses due to probable arterial rigidity. However, those 3 subjects with ABI >1.5 at baseline were not among those 5 with ABI >1.5 at 5 years. Similarly, those 5 with ABI >1.5 at 5 years were not among the 8 with ABI >1.5 at 12 years.

Figure 7 presents the frequency distributions of the ABI measured at each time point. It illustrates a negative skew of the ABI distributions which increases at each time point reflecting the presence and progression of peripheral atherosclerosis over time. In total, 747 subjects had valid ABI measurements at all three clinical examinations and their distributions were similar to those of subjects with measurement of ABI at each time point as shown in Figure 8.

5.4.2 Mean change in ABI after 5 and after 12 years

The mean ABI declined over time (Figure 9). This drop was similar for all subjects measured at each time point and in the subgroup of subjects who had an ABI measured at all three time points (Table 26). In fact, the mean (SD) change from baseline to 5 years was -0.04 (0.18) in those 1,075 subjects who had measurement of ABI at baseline and 5 years and -0.03 (0.17) in 747 subjects who had ABI measurements at baseline, 5 and 12 years. The mean (SD) change from baseline to 12 years examination was -0.06 (0.19) and -0.07 (0.19) in those 1,075 subjects who had measurement of ABI at baseline and 12 years and in those subjects who had ABI measurements at baseline, 5 and 12 years, respectively. (Table 26) All changes were statistically significant ($p < 0.001$).

Figure 10 shows the mean ABI (95% CI) for males and females separately. Females had consistently lower mean (SD) ABI at each time point (1.04 (0.13) at baseline, 1.01 (0.15) at 5 years, 0.98 (0.16) at 12 years) compared to males (1.04 (0.13) at baseline, 1.01 (0.15) at 5 years, 0.98 (0.16) at 12 years). However, there were no statistically significant differences in mean ABI change after 5 and after 12 years between males and females. For the 382 females who have measures at all time points the mean change was -0.03 (0.16) after 5 years and -0.06 (0.18) after 12 years. On the other hand, the 365 males with ABI measurements at all 3 time points had mean change (SD) -0.04 (0.17) after 5 years and -0.06 (0.18) after 12 years.

Finally, among the 747 subjects with valid ABI measurements across all clinical examinations, 10.3%, 16.3% and 22.6% had $ABI \leq 0.9$ at baseline, 5 years and 12

years respectively. Also, 670 of the 747 subjects had normal ABI at baseline (ABI >0.9) and of those the ABI declined below 0.9 in 89 (13.3%) at 5 year examination and in 124 (18.5%) at 12 years examination.

Figure 7 Frequency distribution of ankle brachial index (ABI) of all subjects who participated at baseline, 5 years and 12 years examination

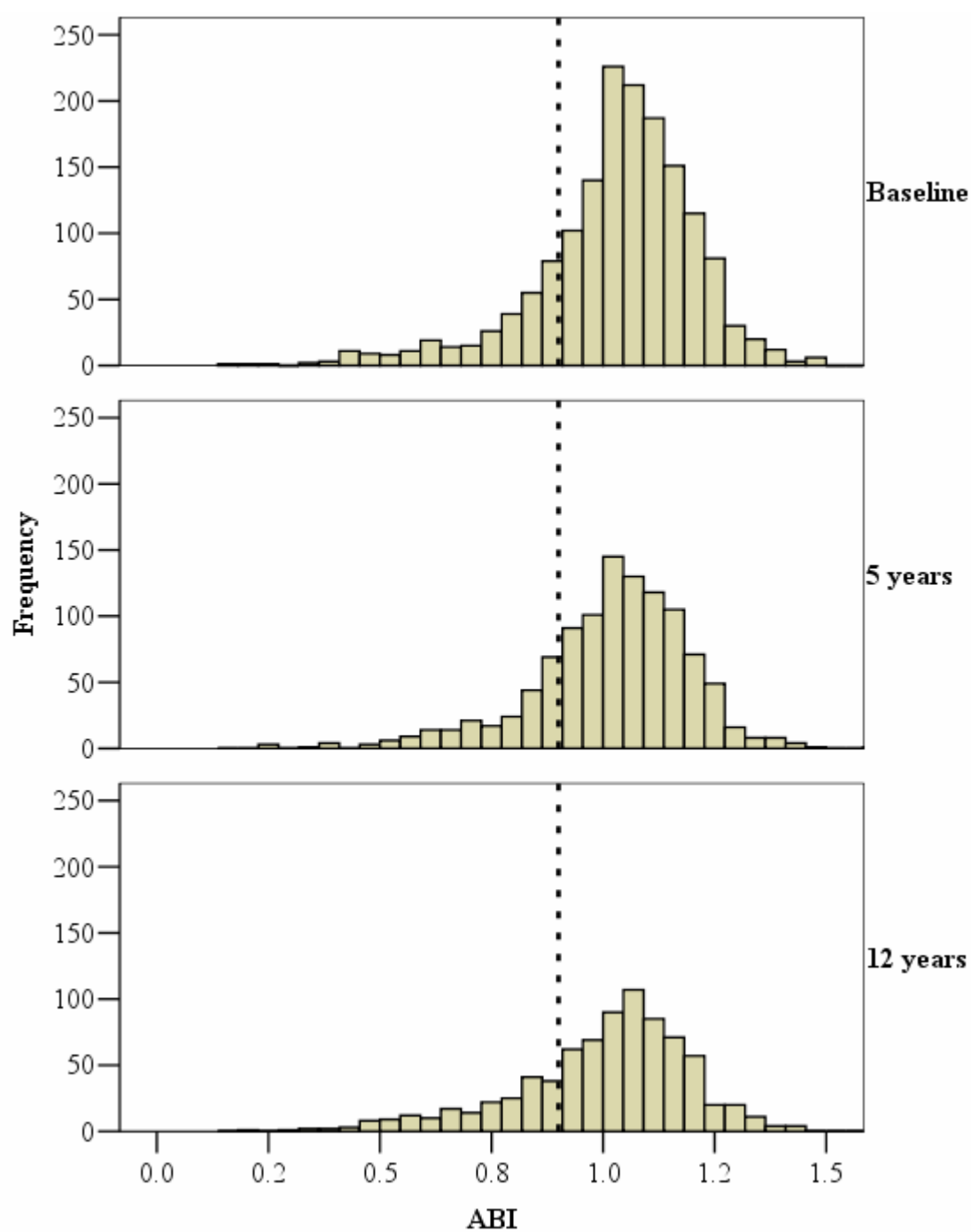


Figure 8 Frequency distribution of ankle brachial index (ABI) at baseline, 5 years and 12 years examination of the 747 subjects who participated in all examinations

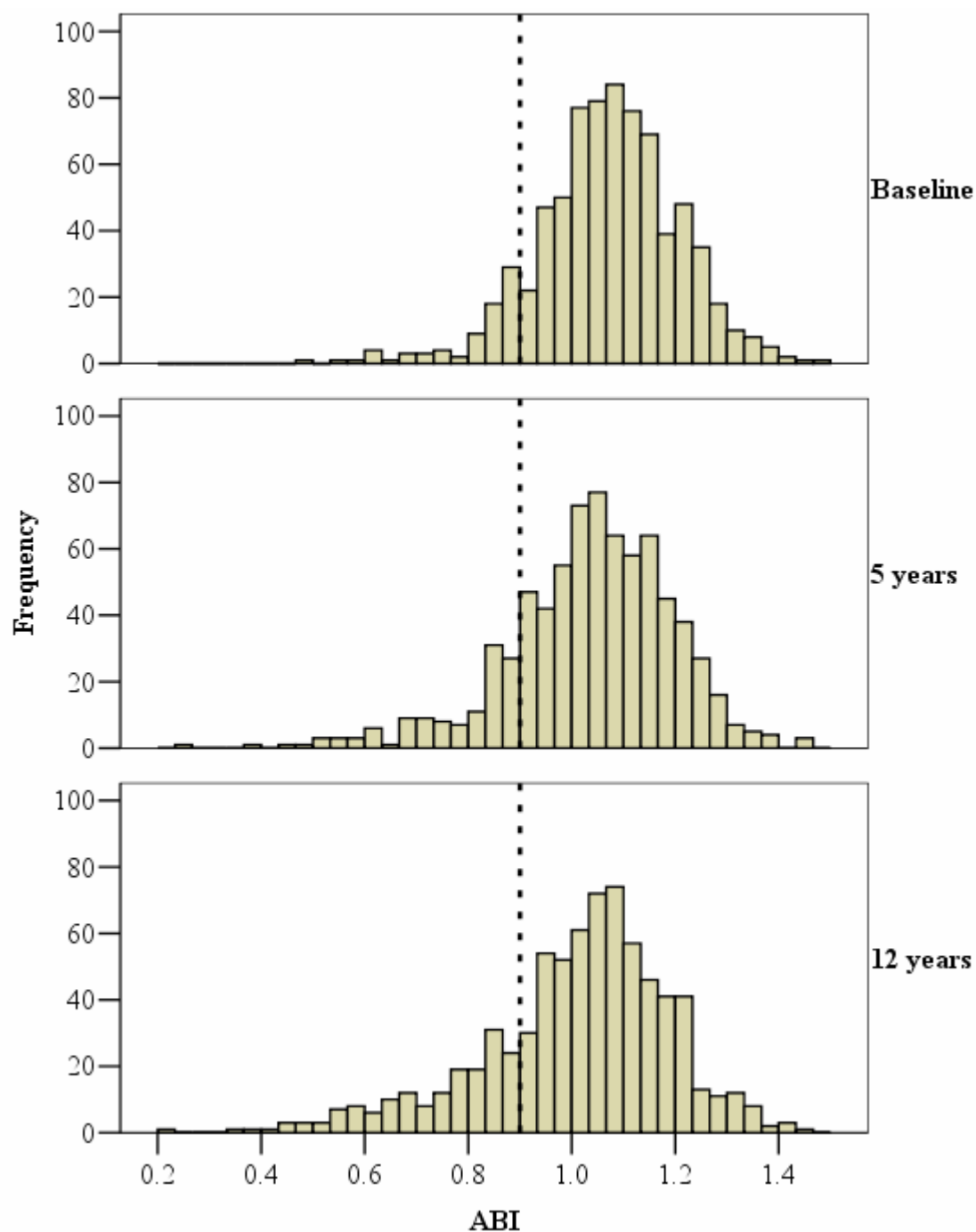


Figure 9 Mean ankle brachial index (ABI) (95% CI) of the 754 subjects who had measures of ABI at baseline, 5 and 12 years examination

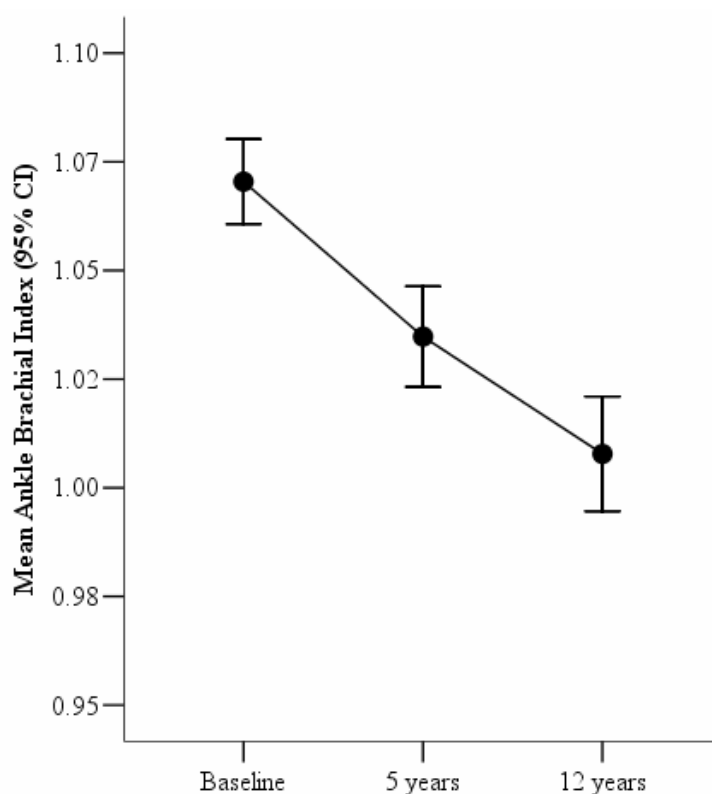
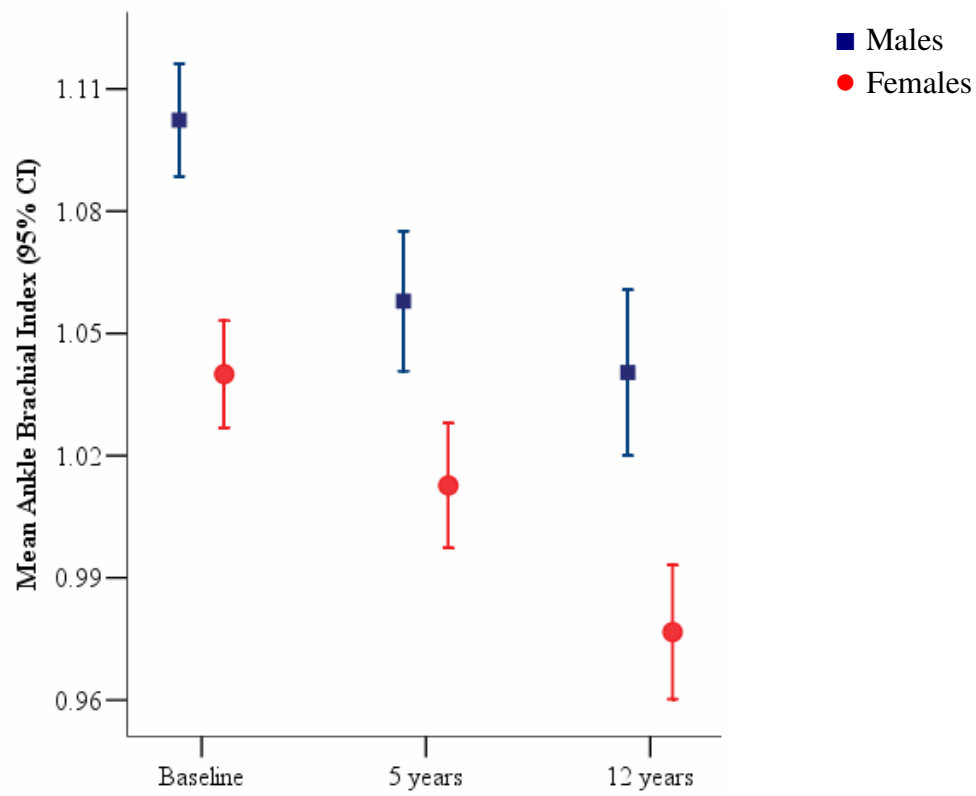


Table 26 Mean (SD) ankle brachial index (ABI) and mean (SD) ABI change between baseline, 5 years and 12 years of follow up both in subjects with ABI at each time point and in 747 subjects with an ABI at all time points

	Subjects with ABI at each time point		Subjects with ABI at all time points	
	N	Mean (SD)	N	Mean (SD)
ABI baseline	1,579	1.03 (0.18)	747	1.07 (0.14)
ABI 5 years	1,076	1.02 (0.17)	747	1.04 (0.16)
ABI 12 years	805	1.00 (0.19)	747	1.01 (0.19)
ABI change at 5 years	1,075	-0.04 (0.18)**	747	-0.03 (0.17)**
ABI change at 12 years	804	-0.07 (0.18)**	747	-0.06 (0.18)**

** p value for change ≤ 0.001

Figure 10 Mean ankle brachial index (ABI) (95% CI) of the 365 males and 382 females that had measures of ABI at baseline, 5 and 12 years examination



5.5 Cardiovascular risk factors and progressive peripheral atherosclerosis

This part describes the associations between cardiovascular risk factors measured at baseline and progression of peripheral atherosclerosis measured by the ABI decline after 5 and after 12 years of follow up.

5.5.1 Cardiovascular risk factors and baseline ABI

Traditional cardiovascular risk factors including cholesterol, ratio of total to HDL cholesterol and pack years of smoking were strongly and significantly ($p < 0.001$) associated with baseline ABI. Pearson correlation coefficients are listed in Table 27. Also, baseline ABI was lower in subjects with diabetes ($p < 0.001$) and mean (SD) 0.97 (0.26) compared to 1.04 (0.17) in subjects with normal glucose levels. Mean (SD) ABI was 0.98 (0.19) in subjects who were self-reported as having no physical activity, 1.02 (0.18) in subjects with light activity, 1.04 (0.18) in subjects with moderate activity and 1.07 (0.18) in subjects with strenuous activity. This decrease in ABI across decreasing physical activity categories had a statistically significant trend (p for trend < 0.001). No clear associations with BMI were observed.

5.5.2 Cardiovascular risk factors and ABI decline after 5 and after 12 years

Conventional cardiovascular risk factors were associated with ABI measured at 5 and 12 years and with ABI decline (Table 27, Table 28). Table 28 lists the correlation coefficients adjusted for baseline ABI between risk factors and ABI after 5 and 12

years. Cholesterol levels and smoking were significantly associated with decline in ABI. However, BMI did not show any significant correlations. Subjects with diabetes had mean (95% CI) ABI change after 12 years -0.10 (-0.16, -0.03) and those without -0.07 (-0.08, -0.05) (p for difference not significant). Table 29 presents the results of linear regression for ABI change after 12 years adjusted for baseline ABI. In univariable analysis, age, sex, total/ HDL cholesterol ratio, smoking and baseline CVD were significantly associated with ABI decline. Most of these associations were strengthened in multivariable (adjusting for all factors) analysis. Similar patterns were seen in regression analyses with ABI change after 5 years as the outcome variable. However, due to smaller mean ABI change at 5 years the effect of each predictor variable was reduced.

Table 27 Pearson correlation coefficients between baseline cardiovascular risk factors and ankle brachial index (ABI) measured at baseline, 5 and 12 years after follow up

	ABI baseline	ABI 5 years	ABI 12 years
Age (years)	-0.18	-0.19	-0.21
Pack years smoking ($\sqrt{}$)	-0.22	-0.09	-0.17
BMI (kg/m^2)	0.02	0.05	0.01
Total cholesterol (mmol/L)	-0.12	-0.15	-0.16
HDL cholesterol (mmol/L)	0.05	-0.04	0.03
Total/ HDL cholesterol	-0.10	-0.06	-0.12

Correlation coefficient in bold are significant at 0.01 level.

Table 28 Pearson correlation coefficients adjusted for baseline ankle brachial index (ABI) between baseline cardiovascular risk factors and ABI change after 5 and after 12 years of follow up

	ABI change after 5 years	ABI change after 12 years
Age (years)	-0.12	-0.20
Pack years smoking ($\sqrt{}$)	0.01	-0.11
BMI (kg/m^2)	0.06	0.05
Total cholesterol (mmol/L)	-0.10	-0.08
HDL cholesterol (mmol/L)	-0.04	0.03
Total/ HDL cholesterol	-0.02	-0.08

Correlation coefficient in bold are significant at 0.01 level

Table 29 Regression coefficient (95% CI) of cardiovascular risk factors for change in ankle brachial index (ABI) after 12 years

	ABI change after 12 years adjusted for baseline ABI	
	Univariable	Multivariable
Age (years)	-0.006 (-0.009, -0.004)**	-0.006 (-0.009, -0.004)**
Sex (males vs. females)	-0.031 (-0.056, -0.006)**	-0.064 (-0.093, -0.040)**
Pack-years smoking (top vs. bottom tertile) [†]	-0.038 (-0.057, -0.014)**	-0.052 (-0.075, -0.028)**
BMI (top vs. bottom tertile) [†]	0.005 (-0.003, 0.015)	0.008 (0.0001, 0.020)*
Diabetes (yes vs. no)	-0.034 (-0.101, 0.033)	-0.031 (-0.097, 0.034)
Total/ HDL cholesterol (top vs. bottom tertile) [†]	-0.014 (-0.024, -0.004)*	-0.016 (-0.028, 0.005)**
MI, stroke or angina (yes vs. no)	-0.076 (-0.119, -0.033)**	-0.052 (-0.093, -0.011)*
Physical activity (strenuous vs. no activity)	0.003 (-0.024, 0.086)	0.010 (-0.046, 0.066)

[†] variables have been fitted as continuous in the model and regression coefficients have been multiplied by the inter-tertile range (difference between the bottom and top tertile) to represent difference between top and bottom tertile of the distribution.

** p value significant at 0.01 level, * p value significant at 0.05 level.

5.6 Inflammatory markers and progressive peripheral atherosclerosis

This part describes the associations between inflammatory markers measured at baseline and progression of peripheral atherosclerosis measured with ABI decline after 5 and after 12 years of follow up.

5.6.1 Associations with baseline ABI

Baseline ABI was negatively associated with levels of CRP, IL-6 and ICAM-1 ($r = -0.17$ and corresponding p value < 0.001 using transformed distributions).

VCAM-1 levels were modestly associated with baseline ABI ($r = -0.06$ and corresponding p value $= 0.04$ using transformed distributions) and E-selectin did not show any significant associations.

5.6.2 Univariable associations with ABI after 5 and 12 years

The mean ABI change from baseline to 5 and 12 years of follow up by tertiles of inflammatory marker levels is demonstrated graphically on Figure 11. Subjects with CRP, IL-6, ICAM-1 and VCAM-1 in the top tertile had a greater ABI decline by 5 years compared to subjects in the bottom tertile. This difference became more prominent after 12 years of follow up. In fact, subjects in the bottom tertiles of CRP and IL-6 did not show any significant change in mean ABI between 5 and 12 years, whereas those in the middle and top tertiles of CRP and IL-6 showed further decline in mean ABI (mean (SD) ABI change -0.02 (0.19) and -0.04 (0.20) for top tertiles of CRP and IL-6 respectively). Substantial differences were also observed for ICAM-1.

After 12 years, the mean change (SD) in ABI from baseline for subjects in the bottom ICAM-1 tertile was -0.04 (0.18) while for those in the top tertile was -0.11 (0.21). There was no evidence for a relationship between ABI change and E-selectin tertiles.

5.6.3 Multivariable associations with ABI after 5 and 12 years

Separate linear regression analysis was performed for ABI change from baseline to 5 years and from baseline to 12 years with each inflammatory marker as a predictor variable (Table 30). CRP and IL-6 were significant predictors of ABI change at 5 years (CRP: $p = 0.03$, IL-6: $p \leq 0.001$) and at 12 years of follow up (CRP: $p = 0.002$, IL-6: $p \leq 0.001$) in analysis adjusted for both age and for baseline ABI. Little change in these associations was found after adjusting for baseline ABI and risk factors: age, sex, pack years of smoking, diabetes, total/ HDL cholesterol ratio, BMI, physical activity and history of CVD. However, CRP had borderline non-significant ($p = 0.08$) associations with ABI at 5 years in risk factor adjusted analysis. ICAM-1 was significantly inversely associated with ABI change at 5 years ($p = 0.02$) and at 12 years follow up ($p \leq 0.001$) in analysis adjusted for age and baseline ABI. On further adjustments for baseline risk factors, ICAM-1 remained an independent predictor of ABI change at 12 years ($p = 0.01$) only.

To compare the effects of each marker on ABI change standardised regression coefficients representing the differences in ABI decline between the top and bottom tertiles of each marker are shown in Table 30. The magnitude of this difference up to 5 years of follow up was greatest for IL-6. However, at 12 years, increased levels of

CRP, IL-6 and ICAM-1 had effects of similar strength on ABI change. Moreover, to examine the simultaneous effect of all inflammatory markers on ABI change at 5 and 12 years, linear regression was used with all inflammatory markers and baseline risk factors simultaneously entered into the model. In this final analysis, IL-6 was the only significant inflammatory marker that independently predicted ABI change at 5 years ($p=0.002$) and at 12 years ($p=0.02$) and the difference (95% CI) in ABI decline between subjects in the top and bottom tertiles of IL-6 was -0.017 (-0.120, -0.008) and -0.016 (-0.034, -0.001) at 5 and 12 years respectively (Table 31).

Finally, the possible effect modification between each marker and sex or age was tested by fitting interaction terms for age or sex and each inflammatory marker. None of the interaction terms tested were statistically significant.

5.6.4 Repeated measures analysis

The aforementioned analysis was also confirmed by mixed model analysis for repeated measures of ABI. In this analysis, only subjects with ABI measurements at all three time points were included. The interaction term of inflammatory markers with follow up time (baseline, 5 or 12 years) was examined. A compound symmetry covariance structure was used since more complex patterns did not lead to any significant improvements in the likelihood function. Using this covariance pattern, levels of IL-6 and ICAM-1 showed a significant interaction term with time (baseline, 5 and 12 years) in both age and sex adjusted ($p=0.02$ and $p=0.008$) and in risk factors adjusted analyses ($p=0.03$ and $p=0.02$).

5.6.5 Sensitivity analysis

Finally, subgroup analysis of those 1,273 subjects with normal ABI ($ABI > 0.9$) and no PAD at baseline was performed. In this subgroup, the odds ratio for transition from normal ($ABI > 0.9$) to low ABI ($ABI \leq 0.9$) was calculated. Results are listed in Table 32. The odds ratios were calculated for each inflammatory marker unadjusted and adjusted for age and sex and then for cardiovascular risk factors. Table 32 lists the odds ratios standardised to represent differences between top and bottom tertile of each marker for crude and age and sex adjusted analyses. Crude odd ratios for CRP, IL-6 and ICAM-1 were significant showing that people with normal ABI but with any of these markers in the top tertile were at increased risk of having an $ABI \leq 0.9$ after 5 and 12 years. However, subsequent adjustments for risk factors attenuated the odds ratios which all became non-significant, probably due to the small sample size of this analysis.

Figure 11 Mean (\pm 1SE) of ankle brachial index (ABI) change from baseline to 5 years and to 12 years of follow up across tertiles of C-reactive protein (CRP), interleukin-6 (IL-6), soluble intercellular adhesion molecule-1 (ICAM-1), soluble vascular adhesion molecule-1 (VCAM-1) and E-selectin

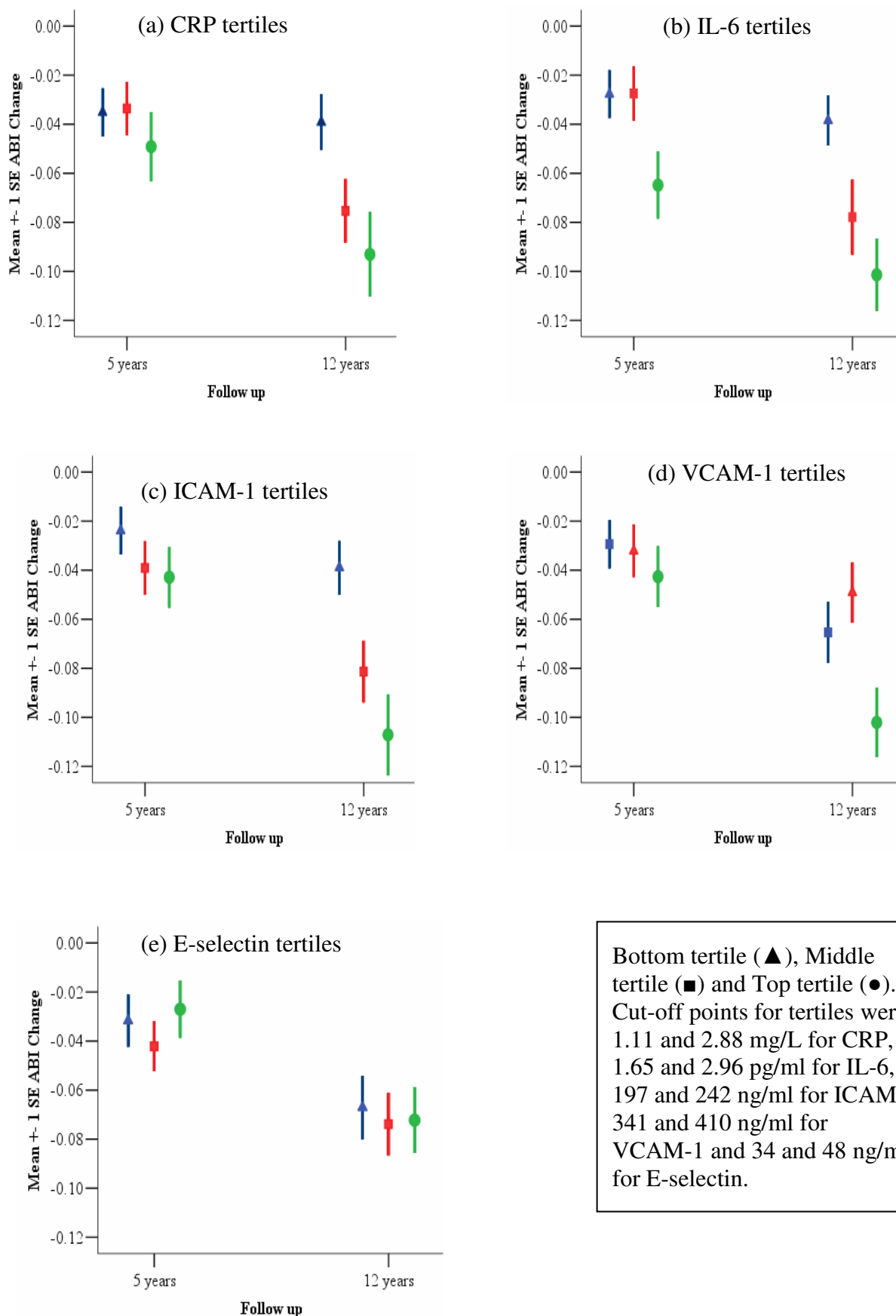


Table 30 Multiple regression for ankle brachial index (ABI) change from baseline to 5 years and to 12 years of follow up and inflammatory markers

Standardised difference (95% CI) in ABI change between the top and bottom tertiles of inflammatory markers [†]		
	Baseline to 5 years	Baseline to 12 years
Adjusted for baseline ABI and age		
CRP	-0.013 (-0.025, -0.001)*	-0.022 (-0.036, -0.008)**
IL-6	-0.018 (-0.028, -0.009)**	-0.023 (-0.034, -0.011)**
ICAM-1	-0.010 (-0.017, -0.002)*	-0.021 (-0.031, -0.011)**
VCAM-1	-0.005 (-0.014, 0.004)	-0.010 (-0.021, 0.001)
E-selectin	-0.001 (-0.011, 0.008)	-0.009 (-0.021, 0.002)
Adjusted for baseline ABI, risk factors and CVD[§]		
CRP	-0.011 (-0.024, -0.001)*	-0.018 (-0.034, -0.004)**
IL-6	-0.017 (-0.027, -0.007)**	-0.019 (-0.031, -0.007)**
ICAM-1	-0.006 (-0.014, 0.003)	-0.014 (-0.024, -0.003)**
VCAM-1	-0.004 (-0.014, 0.005)	-0.008 (-0.019, 0.002)
E-selectin	-0.003 (-0.013, 0.006)	-0.011 (-0.022, 0.0004)
CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1.		
[†] Regression coefficients have been multiplied by the inter-tertile range (difference between the logarithmically transformed bottom and top tertile).		
** p value significant at 0.01 level, * p value significant at 0.05 level.		
[§] adjusted for age, sex, pack years of smoking, diabetes, BMI, total/ HDL cholesterol ratio, physical activity and history of CVD.		

Table 31 Multiple regression for ankle brachial index (ABI) change from baseline to 5 years and to 12 years of follow up and inflammatory markers entered simultaneously in the model

Standardised difference (95% CI) in ABI change between the top and bottom tertiles of inflammatory markers [†]		
	Baseline to 5 years	Baseline to 12 years
Adjusted for baseline ABI and age		
CRP	0.002 (-0.013, 0.017)	0.001 (0.017, 0.019)
IL-6	-0.020 (-0.035, 0.005)**	-0.019 (0.036, -0.002)*
ICAM-1	-0.003 (-0.015, 0.009)	-0.022 (0.037, -0.007)
VCAM-1	-0.003 (-0.018, 0.011)	0.0004 (0.017, 0.018)
E-selectin	0.012 (-0.002, 0.025)	0.006 (0.011, 0.022)
Adjusted for baseline ABI, risk factors and CVD[§]		
CRP	-0.001 (0.017, 0.015)	0.001 (0.018, 0.020)
IL-6	-0.018 (0.033, 0.003)*	-0.016 (0.034, -0.001)*
ICAM-1	0.002 (0.011, 0.015)	-0.014 (0.030, 0.003)
VCAM-1	-0.004 (0.019, 0.010)	-0.001 (0.019, 0.017)
E-selectin	0.006 (0.009, 0.020)	0.0002 (0.018, 0.017)

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1.

[†]Regression coefficients have been multiplied by the inter-tertile range (difference between the logarithmically transformed bottom and top tertile).

** p value significant at 0.01 level, * p value significant at 0.05 level.

[§] adjusted for age, sex, pack years of smoking, diabetes, BMI, total/ HDL cholesterol ratio, physical activity and history of CVD.

Table 32 Odds ratios for transition from normal baseline ankle brachial index (ABI) (ABI>0.9) to low ABI (ABI ≤ 0.9) at 5 and 12 years

Odds ratio [†] (95% CI) for ABI transition from normal to low between top and bottom tertile of each marker		
	Baseline to 5 years	Baseline to 12 years
Crude		
CRP	1.22 (1.07, 1.52)**	1.33 (1.06, 1.68)**
IL-6	1.28 (1.07, 1.52)**	1.22 (1.01, 1.47)*
ICAM-1	1.18 (1.03, 1.36)*	1.24 (1.06, 1.45)**
VCAM-1	1.07 (0.89, 1.28)	1.06 (0.87, 1.29)
E-selectin	1.05 (0.88, 1.26)	1.03 (0.84, 1.25)
Adjusted for age and sex		
CRP	1.11 (0.89, 1.38)	1.23 (0.97, 1.57)
IL-6	1.25 (1.04, 1.51)*	1.17 (0.96, 1.43)
ICAM-1	1.14 (0.99, 1.32)	1.22 (1.03, 1.43)
VCAM-1	0.99 (0.82, 1.19)	0.97 (0.79, 1.20)
E-selectin	1.10 (0.91, 1.33)	1.09 (0.88, 1.34)

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1.

[†]Regression coefficients have been multiplied by the inter-tertile range (difference between the logarithmically transformed bottom and top tertile).

** p value significant at 0.01 level, * p value significant at 0.05 level.

5.7 Haemostatic markers and progressive peripheral atherosclerosis

This part describes the associations between haemostatic markers measured at baseline and progression of peripheral atherosclerosis measured with ABI decline after 5 and after 12 years of follow up.

5.7.1 Associations with baseline ABI

Transformed levels of fibrinogen and D-dimer were strongly and negatively associated with baseline ABI with Pearson correlation coefficients -0.23 ($p < 0.001$) and -0.26 ($p < 0.001$) respectively. Levels of t-PA and vWF were also negatively associated with baseline ABI (Pearson correlation coefficients -0.10 and $p < 0.001$ using the transformed distributions) whereas factor VII, F1+2 and FpA had non-significant associations.

5.7.2 Associations with ABI after 5 and 12 years

The mean ABI change from baseline to 5 and 12 years of follow up by tertiles of haemostatic markers levels is demonstrated graphically in Figure 12. Decline of ABI after 5 years showed weak associations with increasing tertiles of t-PA, vWF, factor VII and FpA but not for fibrinogen, D-dimer and F1+2. The mean change in ABI after 12 years was generally greater in subjects falling in the top tertiles of fibrinogen, D-dimer, t-PA, factor VII and FpA than in subjects falling in the bottom tertiles of these markers. Also, subjects in the top tertile of fibrinogen, D-dimer and t-PA showed substantial drop in their mean ABI from 5 to 12 years compared to

those falling in the top tertile. For example people falling in the bottom tertile of D-dimer at baseline had mean (SD) ABI change -0.03 (0.18) after 5 years and -0.01 (0.20) further mean ABI decline from 5 to 12 years. On the other hand, people falling in the top tertile of D-dimer at baseline had mean (SD) ABI change -0.03 (0.19) after 5 years and -0.06 (0.21) further mean ABI decline from 5 to 12 years.

5.7.3 Multivariable associations with ABI after 5 and 12 years

Separate linear regression analysis for ABI change from baseline to 5 and to 12 years with each haemostatic factor as a predictor variable (Table 33) was performed. To allow comparisons between markers, Table 33 presents the mean difference (95% CI) between the top and bottom tertiles of each marker on ABI change.

None of the haemostatic variables significantly predicted ABI decline after 5 years. Associations were in the expected direction but they did not reach statistical significance. Fibrinogen ($p \leq 0.001$) and D-dimer ($p \leq 0.001$) were significantly associated with ABI change after 12 years in analyses adjusted for baseline ABI and baseline age. However, D-dimer had a marginally greater effect with mean difference (95% CI) between top and bottom tertile of -0.017 (-0.029, -0.006). Moreover, in analyses adjusted for baseline ABI, conventional risk factors (age, sex, BMI, diabetes, pack years of smoking, total/ HDL cholesterol ratio, physical activity) and baseline CVD, D-dimer remained significantly and independently associated with ABI change ($p = 0.003$). Nevertheless, its effect was reduced and the mean (95 % CI)

difference between tertiles was -0.015 (-0.027, -0.004). Fibrinogen had borderline significance ($p=0.05$) in this risk factor adjusted analysis.

Next, the corresponding effect of all haemostatic markers on ABI change at 5 and at 12 years was examined by entering all haemostatic markers simultaneously into the linear regression model (Table 34). In the analysis for change after 12 years, D-dimer ($p=0.015$) and fibrinogen ($p=0.025$), were significantly and independently associated with ABI change. When further adjustments for known risk factors were performed, only D-dimer was significantly associated with ABI change independently of other haemostatic markers and cardiovascular risk factors. Mean (95% CI) difference in ABI change between top and bottom tertiles of D-dimer in this final analysis was -0.021 (-0.038, -0.004). A significant result was also found for FpA in the 5 years analysis. Given the fact that FpA did not show any associations with ABI even in the cross-sectional analysis, this result is probably a statistical artefact.

Finally, the possible effect modification between each haemostatic markers and sex or age was tested by fitting interaction terms for age or sex and each haemostatic factor. None of the interaction terms tested were statistical significant.

5.7.4 Repeated measures analysis

The aforementioned analysis was also confirmed by mixed model analysis of repeated measures of ABI as was previously done for inflammatory markers. In this analysis, only subjects with ABI measurements at all three time points were

included. The interaction term of haemostatic markers with follow up time (baseline, 5 or 12 years) was examined. A compound symmetry covariance structure was used since more complex patterns did not lead to any significant improvements in the likelihood function. Using this covariance pattern, levels of fibrinogen and D-dimer showed a significant interaction with time of ABI measurement (baseline, 5 and 12 years) in both age and sex adjusted ($p=0.01$ and $p=0.006$) and in risk factors adjusted analysis ($p=0.02$ and $p=0.006$).

5.7.5 Sensitivity analysis

Subgroup analysis of those 1,273 subjects with normal ABI ($ABI > 0.9$) and no PAD at baseline was performed. In this subgroup the odds ratio for transition from normal ($ABI > 0.9$) to low ABI ($ABI \leq 0.9$) was calculated. Results in Table 23 show the standardised odds ratios for ABI transition between the top and bottom tertile of each marker. The odds ratios were calculated for each haemostatic factor unadjusted and adjusted for age and sex and then cardiovascular risk factors. Odds ratios of fibrinogen, D-dimer and t-PA were statistically significant for ABI transition from normal to low after 5 and after 12 years. Subsequent adjustments for risk factors attenuated these associations.

Figure 12 Mean (\pm 1SE) of ankle brachial index (ABI) change from baseline to 5 years and to 12 years of follow up across tertiles of fibrinogen, D-dimer, tissue plasminogen activator (t-PA), vonWillebrand Factor (vWF), factor VII, prothrombin fragments 1+2 (F1+2), fibrinopeptide A (FpA)

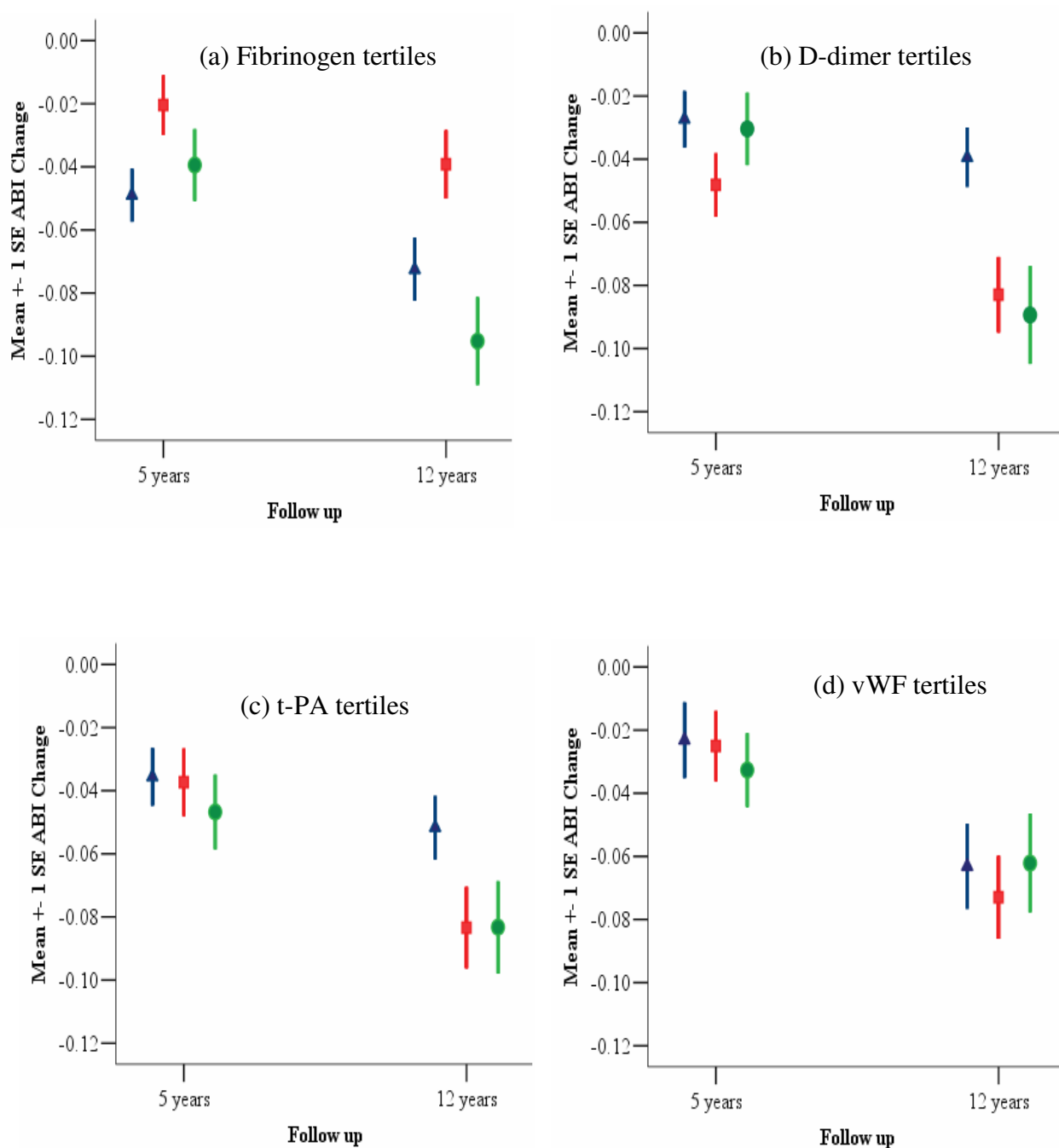
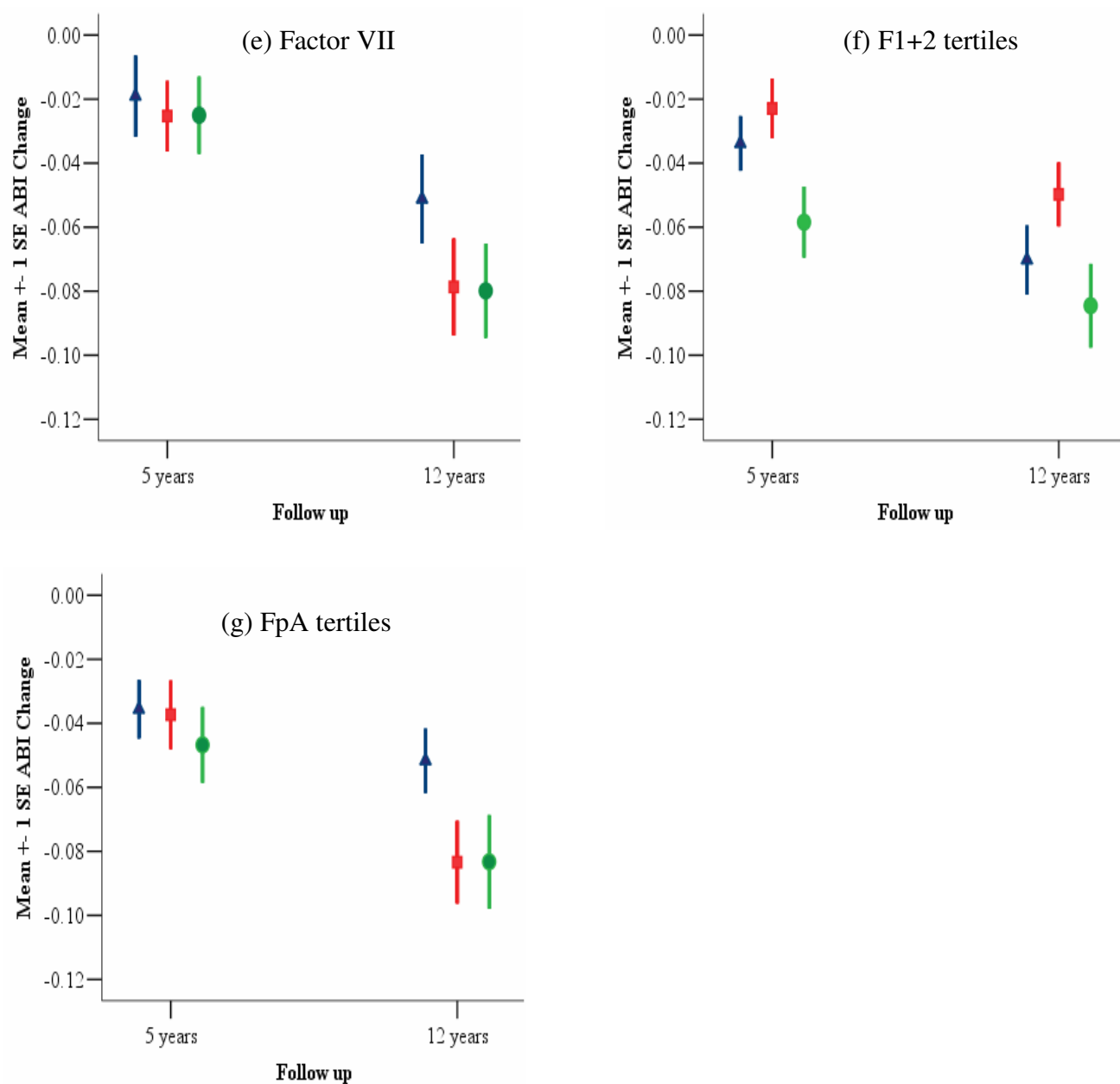


Figure 12 cont.



Bottom tertile (▲), Middle tertile (■) and Top tertile (●). Cut off point for tertiles: 2.38-2.92 g/L for fibrinogen, 67-107 ng/mL for D-dimer, 6.0-8.8 ng/mL for t-PA, 89-129 IU/dl for vWF, 77-105 IU/dl for Factor VII, 1.48-1.95 ng/mL for F1+2, 1.00-1.70 nmol/L for FpA.

Table 33 Multiple regression for ankle brachial index (ABI) change from baseline to 5 years and to 12 years of follow up and haemostatic markers

Standardised difference (95% CI) in ABI change between the top and bottom tertiles of haemostatic factor [†]		
	Baseline to 5 years	Baseline to 12 years
Adjusted for baseline ABI and age		
Fibrinogen	-0.001(-0.009, 0.008)	-0.016 (-0.026, -0.006)**
D-dimer	-0.001 (-0.011, 0.008)	-0.017 (-0.029, -0.006)**
t-PA	-0.003 (-0.013, 0.008)	-0.001 (-0.022, 0.003)
vWF	-0.001 (-0.008, 0.006)	0.005 (-0.004, 0.013)
Factor VII	-0.001 (-0.011, 0.009)	0.006 (-0.006, 0.018)
F1+2	-0.006 (-0.015, 0.003)	-0.008 (-0.019, 0.004)
FpA	-0.009 (-0.020, 0.003)	-0.004 (-0.020, 0.012)
Adjusted for baseline ABI, risk factors and CVD[§]		
Fibrinogen	0.003 (-0.009, 0.008)	-0.010 (-0.021, -0.001)*
D-dimer	-0.001 (-0.011, 0.008)	-0.015 (-0.027, -0.004)**
t-PA	-0.005 (-0.016, 0.006)	-0.008 (-0.022, 0.005)
vWF	0.004 (-0.006, 0.007)	0.007 (-0.001, 0.016)
Factor VII	-0.006 (-0.010, 0.009)	0.009 (-0.003, 0.022)
F1+2	-0.006 (-0.015, 0.003)	-0.008 (-0.019, 0.004)
FpA	-0.009 (-0.021, 0.002)	-0.005 (-0.020, 0.010)

t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

[†]Regression coefficients have been multiplied by the inter-tertile range (difference between the logarithmically transformed bottom and top tertile).

** p value significant at 0.01 level, * p value significant at 0.05 level.

[§] adjusted for age, sex, pack years of smoking, diabetes, BMI, total/ HDL cholesterol ratio, physical activity and history of CVD.

Table 34 Multiple regression for ankle brachial index (ABI) change from baseline to 5 years and to 12 years of follow up with all haemostatic markers entered simultaneously in the model

Standardised difference (95% CI) in ABI change between the top and bottom tertiles of haemostatic factor*		
	Baseline to 5 years	Baseline to 12 years
Adjusted for baseline ABI and age		
Fibrinogen	-0.003 (-0.016, 0.011)	-0.015 (-0.032, -0.002)*
D-dimer	-0.002 (-0.016, 0.012)	-0.020 (-0.037, -0.003)*
t-PA	0.007 (-0.003, 0.017)	0.010 (-0.003, 0.022)
vWF	-0.009 (-0.023, 0.005)	-0.008 (-0.025, 0.009)
Factor VII	0.002 (-0.010, 0.013)	0.012 (-0.002, 0.026)
F1+2	-0.006 (-0.017, 0.004)	-0.002 (-0.016, 0.012)
FpA	-0.023 (-0.042, -0.004)*	-0.002 (-0.026, 0.022)
Adjusted for baseline ABI, risk factors and CVD[§]		
Fibrinogen	-0.002 (-0.016, 0.012)	-0.012 (-0.028, 0.005)
D-dimer	-0.004 (-0.018, 0.010)	-0.022 (-0.040, -0.005)*
t-PA	0.010 (-0.0001, 0.020)	0.012 (-0.0001, 0.025)
vWF	-0.010 (-0.025, 0.005)	-0.005 (-0.023, 0.014)
Factor VII	0.0005 (-0.011, 0.012)	0.010 (-0.004, 0.025)
F1+2	-0.005 (-0.016, 0.006)	-0.002 (-0.016, 0.012)
FpA	-0.024 (-0.043, -0.005)*	-0.002 (-0.025, 0.022)

t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

†Regression coefficients have been multiplied by the inter-tertile range (difference between the logarithmically transformed bottom and top tertile).

** p value significant at 0.01 level, * p value significant at 0.05 level.

§ adjusted for age, sex, pack years of smoking, diabetes, BMI, total/ HDL cholesterol ratio, physical activity and history of CVD.

Table 35 Odds ratio (95% CI) for transition from normal ABI at baseline (ABI>0.9) to low ABI (ABI ≤ 0.9)

Odds ratio (95% CI) for ABI transition from normal to low between top and bottom tertile of each marker		
	Baseline to 5 years	Baseline to 12 years
Crude		
Fibrinogen	1.17 (1.02, 1.36)*	1.25 (1.07, 1.47)**
D-dimer	1.28 (1.09, 1.50)**	1.32 (1.11, 1.57)**
t-PA	1.26 (1.06, 1.50)**	1.20 (1.00, 1.44)*
vWF	1.09 (0.91, 1.29)	0.96 (0.78, 1.17)
Factor VII	1.04 (0.88, 1.25)	1.03 (0.85, 1.24)
F1+2	1.09 (0.92, 1.30)	1.17 (0.98, 1.41)
FpA	1.14 (0.94, 1.38)	1.09 (0.86, 1.37)
Adjusted for age and sex		
Fibrinogen	1.06 (0.91, 1.47)	1.15 (0.97, 1.42)
D-dimer	1.11 (0.93, 1.32)	1.15 (0.95, 1.39)
t-PA	1.22 (1.02, 1.47)	1.17 (0.98, 1.35)
vWF	0.98 (0.82, 1.17)	0.86 (0.69, 1.06)
Factor VII	1.00 (0.83, 1.20)	0.97 (0.80, 1.18)
F1+2	0.98 (0.82, 1.18)	1.07 (0.88, 1.30)
FpA	1.13 (0.93, 1.97)	1.11 (0.87, 1.40)

t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

** p value significant at 0.01 level, * p value significant at 0.05 level.

5.8 Additive effect of inflammatory and haemostatic markers on progressive peripheral atherosclerosis

In this part, the additive effect between inflammatory variables, CRP and IL-6, and haemostatic variables fibrinogen and D-dimer was investigated.

5.8.1 Independent and joint effect of CRP, IL-6, D-dimer and fibrinogen

The previous analyses showed that the inflammatory markers, CRP and IL-6, and the haemostatic markers, fibrinogen and D-dimer, were significant and independent predictors of ABI decline, especially after the 12 years period. These four markers were subsequently compared and their additive effect was further investigated. Table 36 (Model 1) shows the separate effect of each of these four variables on ABI change and the corresponding R^2 in models adjusted for baseline ABI and age and further adjusted for cardiovascular risk factors and baseline CVD. IL-6 had marginally the greatest individual effect, explaining 23% of the variance in ABI change in the multivariable model. All four variables explained 23.4% of the variance in mean ABI change after 12 years of follow up in risk factor adjusted analysis (Table 36 (model 2)). IL-6 was the only one of the four variables that was significantly ($p= 0.03$) associated with ABI changes independently of the other three variables, of cardiovascular risk factors and of baseline CVD.

Then these variables were adjusted for each other and all possible combinations of them as predictors of ABI change were examined. Table 37 lists selected

combinations and shows that the joint effect of CRP and D-dimer on ABI change increased the variance explained by the model from 18.1% (when D-dimer was assessed alone) to 23.3% (Table 37). However, both CRP and D-dimer failed to retain their significant associations when they were adjusted for each other in risk factor adjusted analysis. Fibrinogen did not add to any other variable in any analysis as model 3 and 6 show in Table 37. On the other hand, the combined effect of IL-6 and D-dimer further increased the R^2 to 24 % (Model 4). This was the highest R^2 observed from all models that we examined, despite the fact that D-dimer was not significantly associated with ABI change in this model.

5.8.2 Combined variables

Finally, the combined effect of D-dimer and IL-6 and D-dimer and CRP on ABI change was investigated further since these combinations increased the R^2 of each model. Individuals were divided into 4 mutually exclusive groups according to their baseline levels of D-dimer and IL-6: levels of IL-6 and D-dimer in the top tertile (171 subjects), levels of IL-6 in the top tertile and levels of D-dimer in the middle or bottom tertile (180 subjects), levels of D-Dimer in the top tertile and levels of IL-6 in the middle or bottom tertile (170 subjects) and finally levels of both D-dimer and IL-6 in the middle or bottom tertile (506 subjects). Similarly 4 mutually exclusive groups for CRP and D-dimer were constructed. Here, 134 subjects had both D-dimer and CRP at the top tertile at baseline, 188 CRP only, 158 D-dimer only and 489 subjects had none of these markers at the top tertile of their distribution.

The unadjusted means (\pm 1SE) of ABI change of each of these four groups are presented in Figure 13 and Figure 14. A significant trend with subjects in the top

tertiles of both D-dimer and IL-6 having the greatest ABI decline was found in analysis adjusted for baseline ABI ($p = 0.004$) and further adjusted for cardiovascular risk factors ($p = 0.04$). Combined tertiles of CRP and D-dimer presented a less clear trend which was significant ($p=0.01$) in baseline ABI adjusted analysis only.

Table 36 Predicted mean (95% CI) difference in ABI change between subjects in the top and bottom tertiles of D-dimer, fibrinogen, CRP and IL-6

ABI change after 12 years [†] (N= 547 - 706)				
	Adjusted baseline ABI and age	R ²	Adjusted baseline ABI and baseline risk factors [‡] and CVD at baseline	R ²
Model 1: Each marker individually				
Fibrinogen	-0.016 (-0.026, -0.006)**	13.8%	-0.010 (-0.021, -0.001)*	17.9%
D-dimer	-0.017 (-0.029, -0.006)**	13.4%	-0.015 (-0.027, -0.004)**	18.1%
IL-6	-0.022 (-0.036, -0.008)**	18.3%	-0.018 (-0.034, -0.004)**	23.0%
CRP	-0.023 (-0.034, -0.011)**	16.9%	-0.019 (-0.031, -0.007)**	21.6%
Model 2: All markers simultaneously				
D-dimer	-0.009 (-0.024, 0.005)		-0.009 (-0.023, 0.006)	
Fibrinogen	-0.003 (-0.019, 0.014)		0.006 (-0.010, 0.023)	
IL-6	-0.020 (-0.035, -0.002)*	18.6%	-0.020 (-0.033, -0.002)*	23.4%
CRP	-0.003 (-0.021, 0.016)		-0.002 (-0.021, 0.016)	

CRP: C-Reactive Protein, IL-6: Interleukin-6

[‡] age, sex, pack years of smoking, diabetes, BMI, total/ HDL cholesterol, physical activity and history of CVD.

** p value significant at 0.01 level, * p value significant at 0.05 level.

Table 37 Predicted mean (95% CI) difference in ABI change between subjects in the top and bottom tertiles of D-dimer, fibrinogen, CRP and IL-6

ABI change after 12 years [†] (N= 547 - 706)				
	Adjusted baseline ABI and age	R ²	Adjusted baseline ABI and baseline risk factors [‡]	R ²
Model 3: Fibrinogen and D-dimer simultaneously				
Fibrinogen	-0.012 (-0.023, 0.001)	14.1%	-0.007 (-0.018, 0.005)	18.5%
D-dimer	-0.014 (-0.026, -0.002)*		-0.013 (-0.025, 0.002)*	
Model 4: IL-6 and D-dimer simultaneously				
IL-6	-0.017 (-0.031 -0.004)*	19.3%	-0.014 (-0.028, -0.001)*	24.0%
D-dimer	-0.012 (-0.026, 0.001)		-0.011 (-0.025, 0.002)	
Model 5: CRP and D-dimer simultaneously				
CRP	-0.012 (-0.027, 0.004)	18.4%	-0.007 (-0.024, 0.009)	23.3%
D-dimer	-0.016 (-0.030, -0.002)*		-0.014 (-0.028, 0.0002)	
Model 6: IL-6 and fibrinogen simultaneously				
IL-6	-0.017 (-0.031, -0.004)*	18.5%	-0.017 (-0.031, -0.003)*	23.0%
Fibrinogen	-0.009 (-0.022, 0.004)		-0.003 (-0.016, 0.010)	

CRP: C-Reactive Protein, IL-6: Interleukin-6.

[‡]RF: age, sex, pack years of smoking, diabetes, BMI, total/ HDL cholesterol, physical activity and history of CVD.

** p value significant at 0.01 level, * p value significant at 0.05 level.

Figure 13 Unadjusted mean (\pm 1SE) of ankle brachial index (ABI) change from baseline to 12 years of follow up across combined tertiles of interleukin 6 (IL-6) and D-dimer

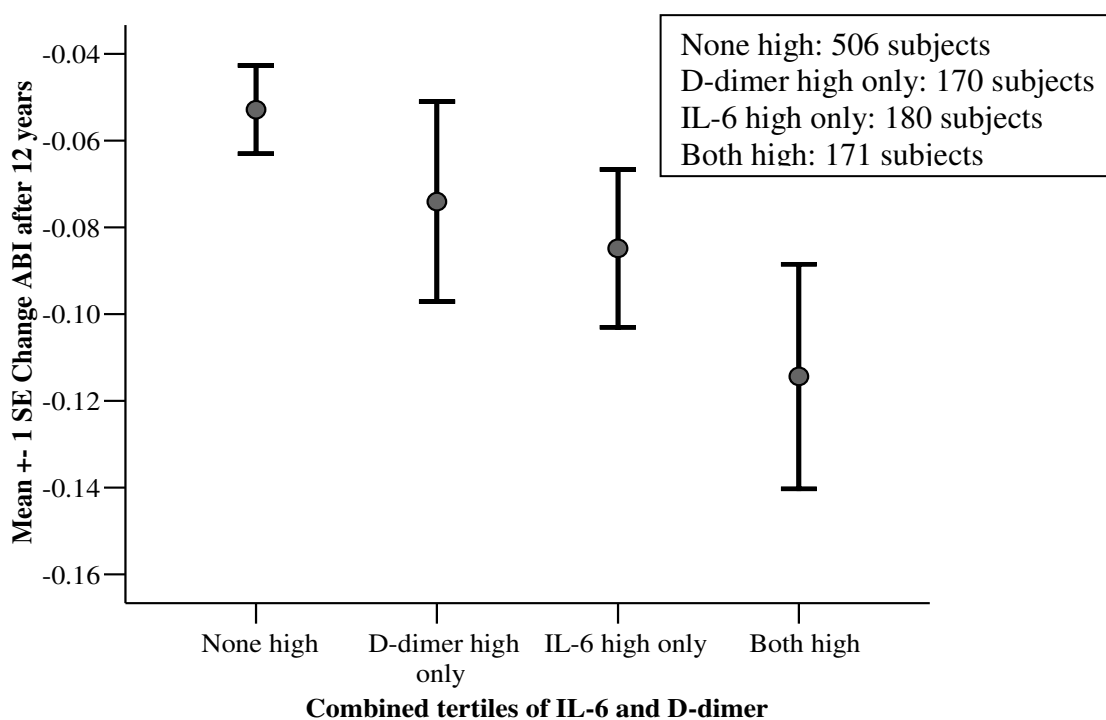
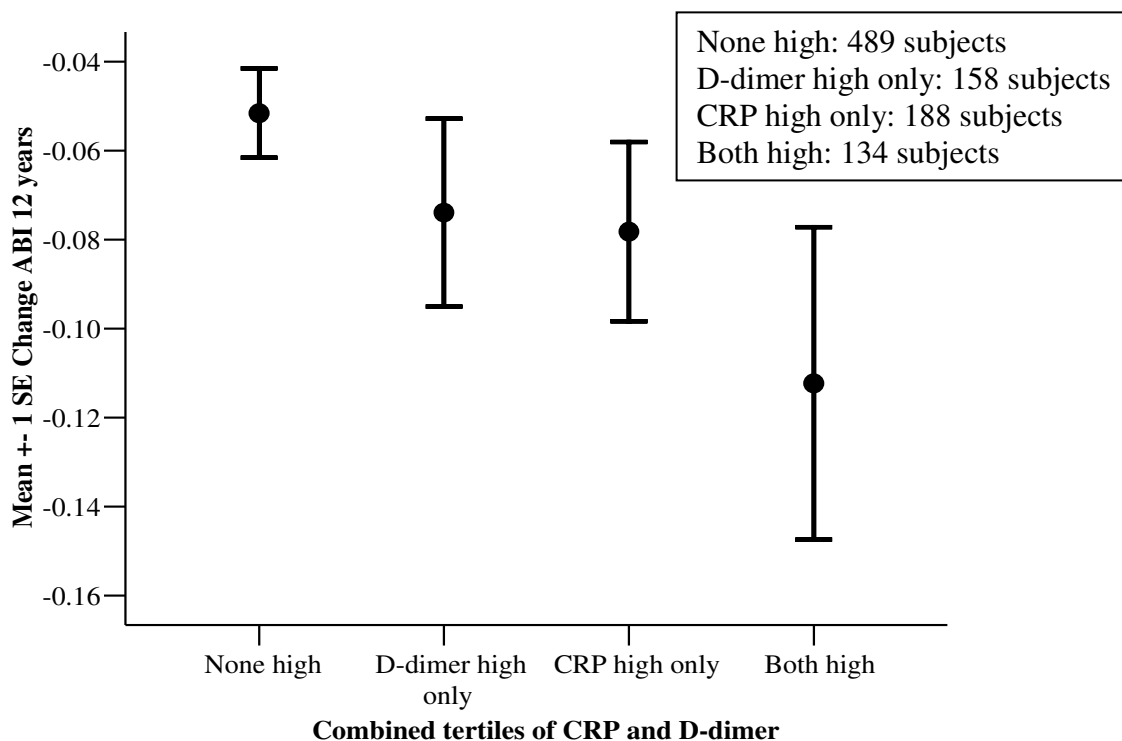


Figure 14 Unadjusted mean (\pm 1SE) of ankle brachial index (ABI) change from baseline to 12 years of follow up across combined tertiles of C-reactive protein (CRP) and D-dimer



5.9 Inflammatory or haemostatic markers and progressive peripheral atherosclerosis according to cardiovascular risk factors status

In this part, the effect of the aforementioned markers was further investigated in relation to baseline cardiovascular risk factors. From the previous analysis, ratio of total/ HDL cholesterol and smoking had greater effect in predicted ABI decline in multivariable analysis than other cardiovascular risk factors. Similarly, D-dimer, IL-6, CRP and to a lesser extent fibrinogen, had significant associations with ABI decline which were independent of conventional risk factors. Therefore, the effect of these markers on ABI change was investigated further according to baseline levels of cholesterol and smoking status.

5.9.1 Cholesterol levels

In order to examine the relative effect of fibrinogen, D-dimer, IL-6 and CRP in individuals with high vs. low cholesterol levels the baseline population was divided into two groups according to their ratio of total cholesterol to HDL cholesterol measured at baseline (below median and above median). The effect of each marker was then examined in each subgroup separately. Subjects with increased CRP levels (top tertile) showed substantial decline in ABI over time (12 years) (mean ABI change -0.12 (0.20)) when they had elevated total/ HDL cholesterol at baseline but not if their ratio levels were below the median (mean (SD) ABI change -0.06 (0.20)) (Figure 15).

The other markers showed comparable associations between increasing tertiles and ABI decline in both groups (total/ HDL cholesterol below median or above median). For example, mean (SD) ABI change after 12 years of follow up for the top tertile of IL-6 was -0.10 (0.17) for individuals with total/ HDL ratio below the median and -0.010 (0.19) for individuals with total/ HDL cholesterol above the median at baseline.

Linear regression with each of these markers (CRP, IL-6, fibrinogen and D-dimer) as predictor variable in the two subgroups confirmed this descriptive analysis. The mean (95% CI) difference in ABI decline between people falling in the top and bottom tertile of CRP at baseline was -0.033 (-0.055, -0.010) ($p=0.004$) if their total/ HDL cholesterol was above the median (analysis was adjusted for baseline ABI and age). On the contrary, if their total/ HDL cholesterol was below the median the effect CRP was not significant and the difference in ABI decline between people falling in the top and bottom tertile of CRP at baseline was 0.001 (-0.018, 0.020) ($p=0.91$) (Figure 16). Fibrinogen showed similar results since it failed to reach statistical significance in the group of people with total/ HDL cholesterol below the median (mean (95% CI) ABI decline between people falling in the top and bottom tertile 0.005 (-0.02, 0.010) $p=0.49$). On the other hand, in people with ratio of total over HDL cholesterol above the median, the mean (95% CI) ABI decline between people falling in the top and bottom tertile was -0.021 (-0.039, -0.007) ($p=0.02$) (analysis was adjusted for baseline ABI and age).

D-Dimer had smaller differences in its effect between people in the below or above the median of total/HDL cholesterol: mean (95% CI) ABI decline between people falling in the top and bottom tertile was -0.010 (-0.024, 0.007) ($p=0.29$) and -0.023 (-0.040, -0.006) ($p=0.01$) respectively (analysis was adjusted for baseline ABI and age).

Finally, for the IL-6, no apparent difference in its effect was found in people with high or low cholesterol ratio at baseline. For example, the mean (95% CI) difference in ABI decline (adjusted for baseline ABI and age) between people falling in the top and bottom tertile of IL6 at baseline was -0.015 (-0.032, 0.002) ($p=0.09$) and -0.019 (-0.038, -0.001) ($p=0.04$) when their total/ HDL cholesterol was above or below the median respectively.

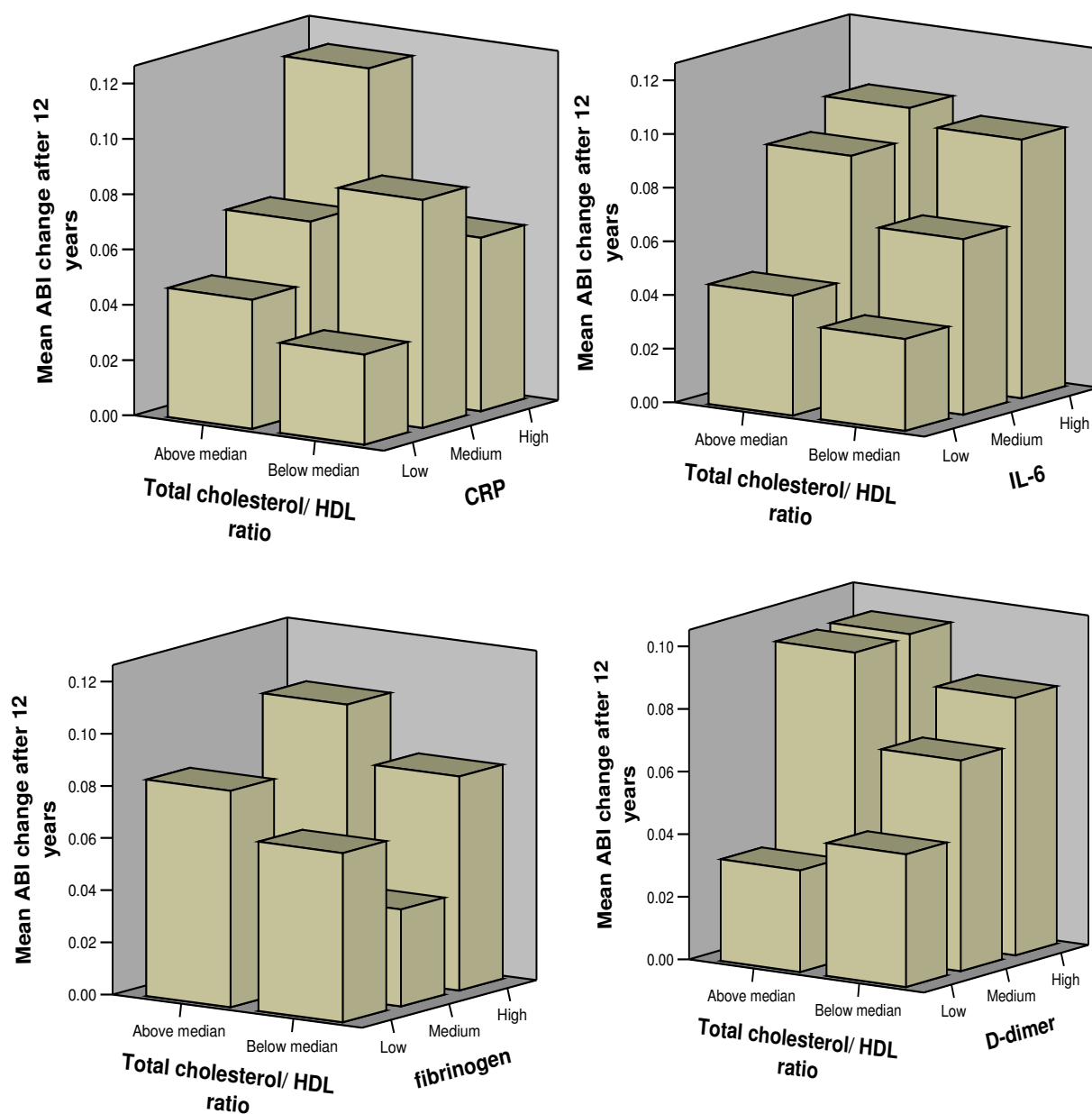
5.9.2 Smoking

When the baseline population was divided into smokers (current and ex) and never smokers there considerable differences were seen in the effect of CRP and fibrinogen on ABI change (Figure 17). Less clear evidence for IL-6 and D-dimer was observed (Figure 17).

All four variables had significant regression coefficients for ABI change after 12 years when analysis was limited to those people who were current smokers or who were ex smokers at baseline. The corresponding regression coefficients representing the difference (95% CI) between top and bottom tertile in ABI change after 12 years of follow up for CRP and fibrinogen were: -0.027 (-0.047, -0.007) ($p=0.01$) and

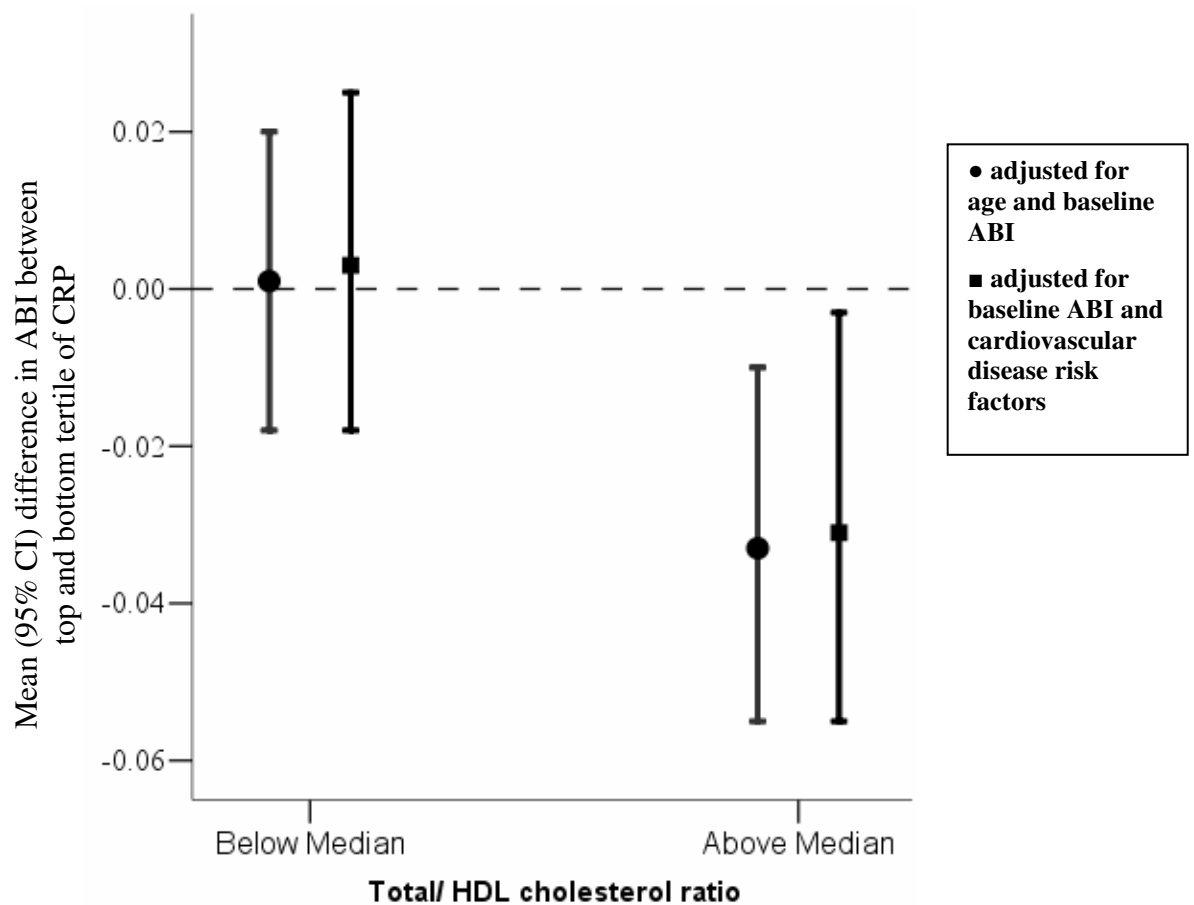
-0.023 (-0.037, -0.009) ($p=0.002$) in analysis adjusted for baseline ABI and age, respectively. On the other hand, in never smokers, the corresponding regression coefficients for CRP and fibrinogen were -0.002 (-0.021, 0.021) ($p=0.85$) and -0.004 (-0.019, 0.011) ($p=0.60$). IL-6 and D-dimer, although had significant and higher regression coefficient in analysis including only smokers they did not display considerable difference between the two groups. In detail, the regression coefficients representing the difference (95% CI) between top and bottom tertile in ABI change after 12 years of follow up for IL-6 and D-dimer were -0.020 (-0.038, -0.002) ($p=0.027$) and -0.017 (-0.033, -0.001) ($p=0.04$) in smokers and -0.012 (-0.030, 0.005) ($p=0.15$) and -0.014 (-0.031, 0.002) ($p=0.10$) in never smokers.

Figure 15 Mean changes in ankle brachial index (ABI) according to baseline levels of total/ HDL cholesterol and C-reactive protein (CRP), interleukin-6 (IL-6), fibrinogen and D-dimer



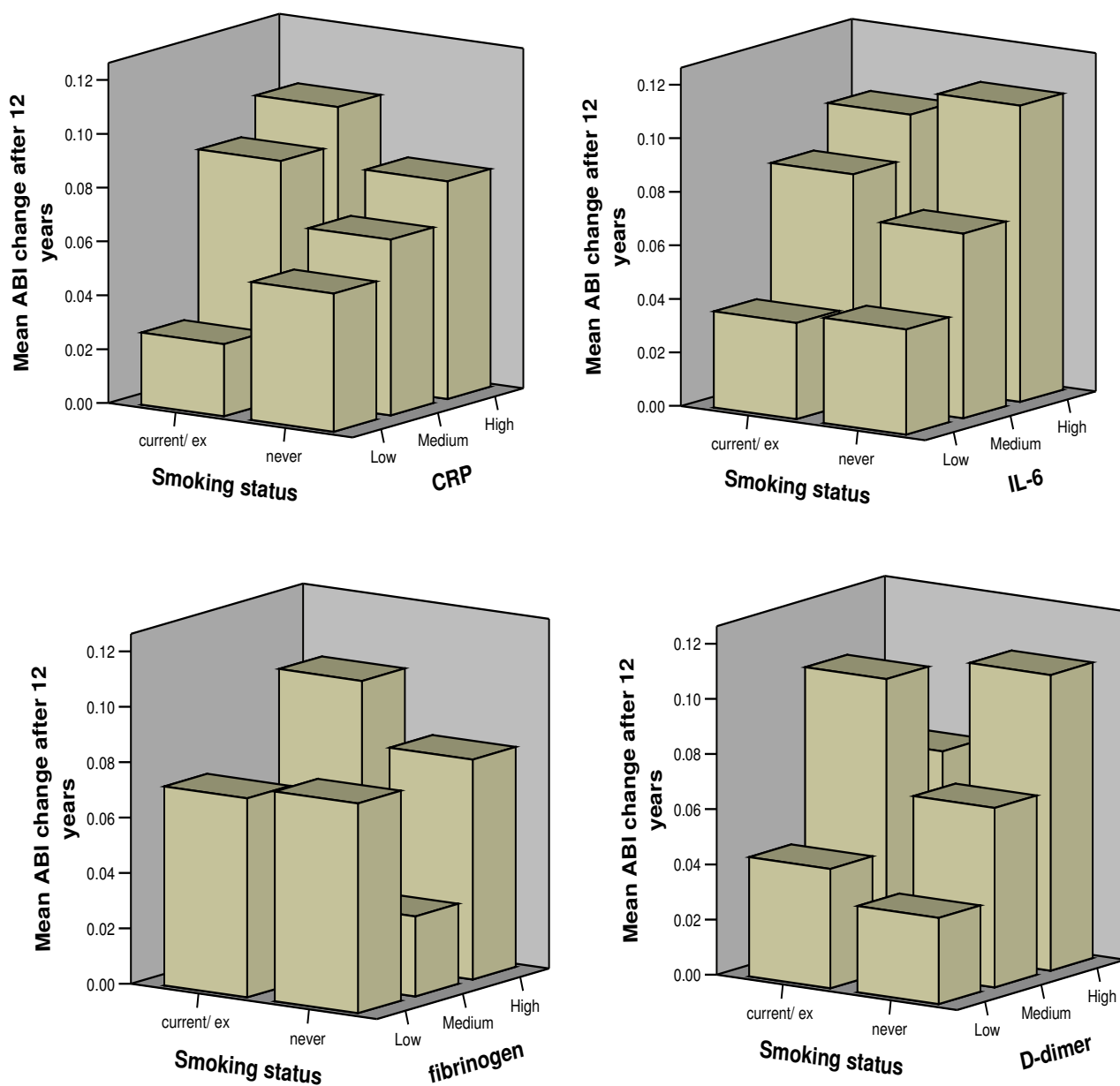
Cut-off point for median total/ HDL cholesterol: 4.96, tertiles of CRP: 1.11 and 2.88 mg/L, IL-6: 1.65 and 2.96 pg/mL, fibrinogen 2.38 and 2.92 g/L and D-dimer: 6.0 and 8.8 ng/mL.

Figure 16 Predicted mean (95% CI) difference in ankle brachial index (ABI) change between subjects in the top and bottom tertiles of C-reactive protein (CRP) according to baseline total/ HDL cholesterol ratio



Cut-off point for median total/ HDL cholesterol: 4.96, tertiles of CRP: 1.11 and 2.88 mg/L.

Figure 17 Mean changes in ankle brachial index (ABI) according to smoking status and C-reactive protein (CRP), interleukin-6 (IL-6), fibrinogen and D-dimer



Never smokers: 605 subjects, current/ ex smokers: 987 subjects, tertiles of CRP: 1.11 and 2.88 mg/L, IL-6: 1.65 and 2.96 pg/mL, fibrinogen 2.38 and 2.92 g/L and D-dimer: 6.0 and 8.8 ng/mL.

5.10 Summary of results

In the aforementioned analysis, inflammatory and haemostatic markers showed strong associations between them and with cardiovascular risk factors and CVD at baseline. These associations were stronger for CRP, IL-6, ICAM-1, fibrinogen, D-dimer and t-PA whereas E-selectin, factor VII, FpA and F1+2 showed in general weak associations.

The study population had a significant ($p < 0.001$) progression of peripheral atherosclerosis after 12 years measured by the decline in ABI (mean (SD) change after 12 years -0.07 (0.18)). From inflammatory markers, CRP ($p < 0.01$), IL-6 ($p < 0.001$) and ICAM-1 ($p < 0.01$) were associated with atherosclerotic progression after 12 years, independently of baseline ABI and of cardiovascular risk factors. Also, from haemostatic markers, fibrinogen ($p = 0.05$) and D-dimer ($p \leq 0.05$) were significantly associated with atherosclerotic progression independently of baseline ABI and cardiovascular risk factors. Moreover, subjects with higher levels of both D-dimer and IL-6 at baseline had the greatest ABI decline. IL-6 showed the stronger independent effect on atherosclerotic progression and retained statistical significance after adjustments for all inflammatory markers and for fibrinogen and D-dimer. The associations of IL-6 with atherosclerotic progression did not seem to differ according to baseline levels of total/HDL cholesterol or smoking. However, the association of CRP with atherosclerotic progression was considerably stronger among those who had high total/ HDL cholesterol or were smokers at baseline.

Chapter six

6 Results: Inflammatory and haemostatic markers and incident PAD and CVD

6.1 Introduction

In this chapter the associations between inflammatory and haemostatic markers with incident PAD were examined. The incidence of PAD over 17 years was described. Associations between conventional cardiovascular risk factors and incident PAD were investigated in univariable and multivariable analyses. Inflammatory and haemostatic markers were examined as risk factors for incident PAD in analyses adjusted for conventional risk factors. Multivariable models with several inflammatory and haemostatic markers and combined variables were performed. In addition, their effect was examined according to cardiovascular risk factors status at baseline. In the second part of this chapter, inflammatory and haemostatic markers were examined as risk factors for incident major CVD, MI or stroke in order to compare their effect on different manifestations of the disease. Analyses were adjusted for conventional cardiovascular risk factors and baseline subclinical CVD. Finally, analyses looking at combined variables, multivariable models and interactions with cardiovascular risk factors status as in incident PAD analysis were performed.

Tables and figures are presented at the end of each section.

6.2 Incident PAD

In this part the incidence of PAD during the follow up period is described.

6.2.1 Incidence of moderate and severe PAD

At baseline, 73 subjects were suffering from PAD diagnosed by the WHO IC questionnaire. From the 1,519 subjects who were free of PAD, 209 developed symptomatic PAD during the subsequent 17 years. Of them, 169 (81%) were diagnosed with moderate PAD (IC only). Moreover, 40 (19%) subjects experienced severe PAD defined as CLI (rest pain, gangrene or ulcer) or intervention (amputation or vascular surgery). In detail, 11 (27%) experienced CLI, 26 (65%) amputation and 9 (22%) vascular surgery, whereas 6 subjects (1.5%) experienced more than one severe PAD event. Finally, of those 40 with severe PAD, 26 (65%) had also been diagnosed with IC.

The mean time to diagnosis of incident PAD for the study population was 84 months (range 7-188 months). Also, from the 209 events of PAD, 113 (54%) have occurred in males and 96 (46%) in females (p for difference =0.33). The Kaplan-Meier (KM) curve for PAD events for males and females separately is shown in Figure 18. The steep drop in the line at 5 and 12 years is due to the fact that all participants completed the WHO IC questionnaire at the 5 and 12 years of follow up and thus many events were recorded at those two time points.

6.2.2 Prevalence of moderate PAD at 5 and 12 years

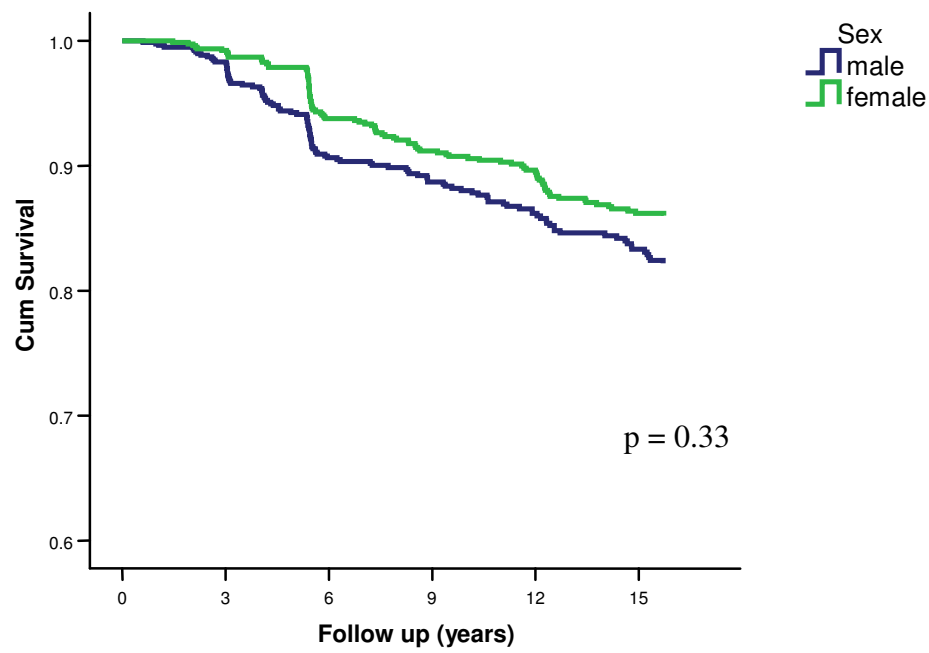
At 5 and 12 years after follow up, participants completed the WHO IC questionnaire.

At 5 years, 1,279 subjects completed the questionnaire and of them 10 (0.8%) had grade I claudication, 19 (1.5%) grade II and 42 (3.3%) probable claudication. The overall IC prevalence was 7.2%. At 12 years, 925 subjects completed the WHO IC questionnaire and the overall disease prevalence was 6.4%. In detail, 14 (1.5%) subjects had grade I claudication, 18 (1.9%) had grade II claudication and 27 (2.9%) had probable claudication.

6.2.3 Comorbidity of PAD with major CVD

From the 73 subjects with IC at baseline, 35 (48%) experienced either a fatal or non-fatal event of MI or stroke. On the other hand, from those 209 subjects who developed new PAD after 17 years of follow up, 89 (43%) subjects also experienced an MI or stroke event.

Figure 18 Survival curves for incident peripheral arterial disease (PAD) over 17 years of follow up for males and females. The p value corresponds to the log rank test



6.3 Traditional risk factors and incident PAD

In this part, the association between traditional cardiovascular risk factors and the incidence of PAD was examined. Throughout this analysis only the 1,519 subjects without baseline PAD were included in the analysis.

6.3.1 Descriptive analysis

Table 38 lists the means (SD) of cardiovascular risk factors measured at baseline in those subjects who developed or did not develop PAD. Subjects who developed PAD were generally older (mean age 65.6 years) and had higher percentage of males (54%) compared to those that did not develop PAD (mean age 64.6 years, 50% males). Moreover, mean levels of total cholesterol, ratio of total/ HDL cholesterol, pack years of smoking and systolic blood pressure and prevalence of hypertension and baseline CVD were all significantly higher at baseline among subjects who developed PAD. On the contrary, levels of HDL cholesterol were significantly lower in those who developed PAD. Moreover, of those who developed PAD, 7.3% suffered from diabetes at baseline compared to 5.5% subjects with diabetes in the healthy group. No statistically significant differences were found for BMI, diastolic blood pressure and physical activity (Table 38).

Then the population was split into those who remained free of PAD, those who developed moderate PAD (IC only) and to those who developed severe PAD (CLI or intervention) and mean levels of cardiovascular risk factors were calculated across disease categories (Table 39). A significant trend across worsening disease categories

was found for HDL cholesterol ($p=0.002$), which had lower levels in the moderate PAD group (mean 1.4 mmol/L) and even lower levels (mean 1.3 mmol/L) compared to the healthy group (mean 1.5 mmol/L). Despite the fact that total cholesterol and total/ HDL cholesterol ratio had higher levels in the disease groups (either moderate or severe PAD) compared to the healthy group, there was no clear trend across worsening disease categories (p for trend $=0.06$). On the other hand, mean systolic blood pressure and percentage of hypertensives were higher in the modest PAD group and even higher in the severe PAD group. However, diastolic blood pressure did not show evidence for a dose response relationship with PAD severity. Similarly, those who developed moderate or severe PAD had 25.4% and 27.5% prevalence of CVD at baseline, respectively. Finally, percentage of diabetes was 6.0% in the moderate PAD group and 13.2% in the group that developed severe PAD.

6.3.2 Univariable associations with incident PAD

Survival curves for incident PAD across levels of most baseline risk factors along with p values for the log rank test are shown in Figure 19. All variables except BMI, diastolic blood pressure and physical activity were associated with incident PAD. For example, older subjects had greater risk of developing PAD and their hazard ratio (95% CI) was 1.28 (1.13, 1.46) ($p < 0.001$) per one age band increase (5 years). Figure 19 shows that the ratio of total/ HDL cholesterol had a clear trend across tertiles in relation to PAD survival and people in the top tertile of total/ HDL ratio had hazard ratio (95% CI) 2.62 (1.82, 3.76) ($p < 0.001$) compared to those in the bottom tertile. Moreover, current or ex smokers, hypertensives and people with increased systolic blood pressure were significantly associated with incident PAD.

Finally, the 204 subjects with history of MI, stroke or angina had a 3 fold increase in the risk of developing PAD (hazard ratio (95% CI) 3.45 (2.52, 4.70) ($p < 0.001$)) compared to those 1,315 subjects who were free of CVD at baseline.

6.3.3 Multivariable associations with incident PAD

Cox regression was used to calculate age and sex adjusted and risk factors-adjusted hazard ratios (95% CI) for PAD and total/ HDL cholesterol, pack years of smoking, hypertension, diabetes, BMI, physical activity and baseline CVD (Table 40).

Total/HDL cholesterol ratio, pack years of smoking, hypertension and baseline CVD were significant predictors of PAD development in both age and sex adjusted and multivariable adjusted analyses ($p < 0.001$). In particular, subjects with baseline CVD had more than a 2-fold increase in their risk for developing a PAD (hazard ratio (95% CI) 2.37 (1.70, 3.31)). Similarly, those with total/ HDL cholesterol ratio or with pack-years of smoking in the top tertile of their distribution had more than 2-fold increase in their risk for incident PAD (hazard ratio (95% CI) 2.30 (1.57, 3.38) and 2.34 (1.67, 3.30), respectively). Total cholesterol and smoking were fitted as 3 level categorical variables to enable comparability with following analysis of inflammatory and haemostatic markers. Models with these variables were examined and results were essentially unchanged. For example, the hazard ratio per 1 SD increase in total/HDL cholesterol was 2.17 (2.00, 2.36) in the multivariable analysis. In addition, when smoking status (ever vs. never smokers) was fitted in the model instead of pack years of smoking, the results were almost the same and the HR (95% CI) for incident PAD in ever compared to never smokers was 1.62 (1.18, 2.22) ($p=0.003$).

The assumption of proportional hazards was tested by stratifying the Cox regression with each covariate and then plotting the log-log plot. All plots did not show serious violations of the proportional hazards assumption since the hazard ratios between different categories seemed constant over time. For example, the log-log plot of total/ HDL cholesterol ratio on Figure 20 shows that the hazard ratio between tertiles of total/ HDL cholesterol was approximately constant over time although the instantaneous hazards varied.

6.3.4 Sensitivity analysis

Similar results, although with wider CI, were found in analyses limited to 1,315 subjects without baseline CVD (MI, stroke or angina). In this subgroup the hazard ratio (95% CI) for total/ HDL cholesterol (top vs. bottom tertile) was 2.57 (1.67, 3.98), for pack years of smoking (top vs. bottom tertile) 2.57 (1.75, 3.79) and for hypertension (yes vs. no) 2.14 (1.50, 3.06).

Furthermore, when the analysis was limited to those 1,273 subjects without baseline evidence of subclinical atherosclerosis (defined by an ABI >0.9) the hazard ratios (95% CI) were also essentially unchanged. For total/ HDL cholesterol (top vs. bottom tertile) the hazard ratio (95% CI) was 2.10 (1.32, 3.33), for pack years of smoking (top vs. bottom tertile) 2.03 (1.36, 3.02), for hypertension (yes vs. no) 1.83 (1.26, 2.66) and for baseline CVD (yes vs. no) 2.74 (1.82, 4.11).

Table 38 Cardiovascular risk factors in subjects who did or did not develop peripheral arterial disease (PAD) over the 17 years of follow up

	Mean (SD)/ Percent		P value for difference
	Did not develop PAD (N=1,310)	Developed PAD (N=209)	
Age (years)	64.6 (5.71)	65.6 (5.30)	0.01
Males	50.4 %	54.1 %	0.32
Smoking			
Never smokers	38 %	27 %	0.002
Ever smokers	62 %	73 %	
Pack years smoking ($\sqrt{}$)	2.8 (2.80)	3.8 (2.93)	< 0.001
BMI (kg/m ²)	25.6 (3.91)	25.7 (3.89)	0.81
Diabetes	5.5 %	7.3 %	0.31
Cholesterol/ HDL ratio	5.1 (1.88)	5.6 (1.72)	< 0.001
Total cholesterol (mmol/L)	7.0 (1.29)	7.2 (0.42)	0.01
HDL cholesterol (mmol/L)	1.5 (0.42)	1.3 (0.34)	< 0.001
Systolic blood pressure (mmHg)	143.3 (23.64)	148.3 (24.27)	0.004
Diastolic blood pressure (mmHg)	77.3 (12.33)	78.0 (12.84)	0.44
Hypertension	67.5 %	51.5 %	< 0.001
Physical activity			
Light	36.5 %	39.7 %	
Moderate	42.4 %	43.1 %	0.44
Strength	11.2 %	7.7 %	
Cardiovascular disease			
Myocardial infarction	3.8 %	7.2 %	0.03
Stroke	2.4 %	4.8 %	0.04
Angina	7.5 %	20.1 %	< 0.001
Any of the above	11.5 %	25.8 %	< 0.001

Table 39 Cardiovascular risk factors in people who did not develop peripheral arterial disease (PAD), who developed moderate PAD and who developed severe PAD

	Mean (SD)/ Percent			p value for trend [†]
	Did not develop PAD (N=1,310)	Developed Moderate PAD (N=168)	Developed Severe PAD (N=40)	
Age (years)	64.6 (5.71)	65.1 (5.35)	67.8 (4.50)	< 0.001
Males	50.4 %	52 %	65 %	0.19
Smoking				
Never smokers	37 %	29 %	25 %	< 0.001
Ever smokers	60 %	71 %	75 %	
Pack years smoking (√)	2.8 (2.80)	3.7 (2.95)	3.9 (2.88)	0.01
BMI (kg/m ²)	25.6 (3.91)	25.6 (3.86)	25.8 (4.07)	0.79
Diabetes	5.5 %	5.9 %	13 %	0.14
Cholesterol/ HDL ratio	5.1 (1.88)	5.6 (1.67)	5.7 (1.93)	0.06
Total cholesterol (mmol/L)	7.0 (1.29)	7.3 (1.45)	6.8 (1.22)	0.43
HDL cholesterol (mmol/L)	1.5 (0.42)	1.4 (0.36)	1.3 (0.26)	0.002
Systolic blood pressure (mmHg)	143.3 (23.64)	147.4 (23.7)	152.2 (26.34)	0.02
Diastolic blood pressure (mmHg)	77.3 (12.33)	78.0 (12.88)	78.0 (12.86)	0.69
Hypertension	67.5 %	68.6 %	75.0 %	< 0.001
Physical activity				
Light	36.5 %	41.4 %	25.0 %	0.01
Moderate	42.4 %	45.0 %	32.5 %	
Strength	11.2 %	7.7 %	7.5 %	
Cardiovascular disease				
Myocardial infarction	3.8 %	7.7 %	5.0 %	0.06
Stroke	2.4 %	3.6 %	10.0 %	0.01
Angina	7.5 %	21.3 %	15.0 %	<0.001
Any of the above	11.5 %	25.4 %	27.5 %	<0.001

[†] For categorical variables p values was calculated with the chi-square test for trend. For continuous variable with ANOVA test for trend.

Figure 19 Survival curves for peripheral arterial disease (PAD) for different levels of baseline cardiovascular risk factors. P values correspond to the log rank test

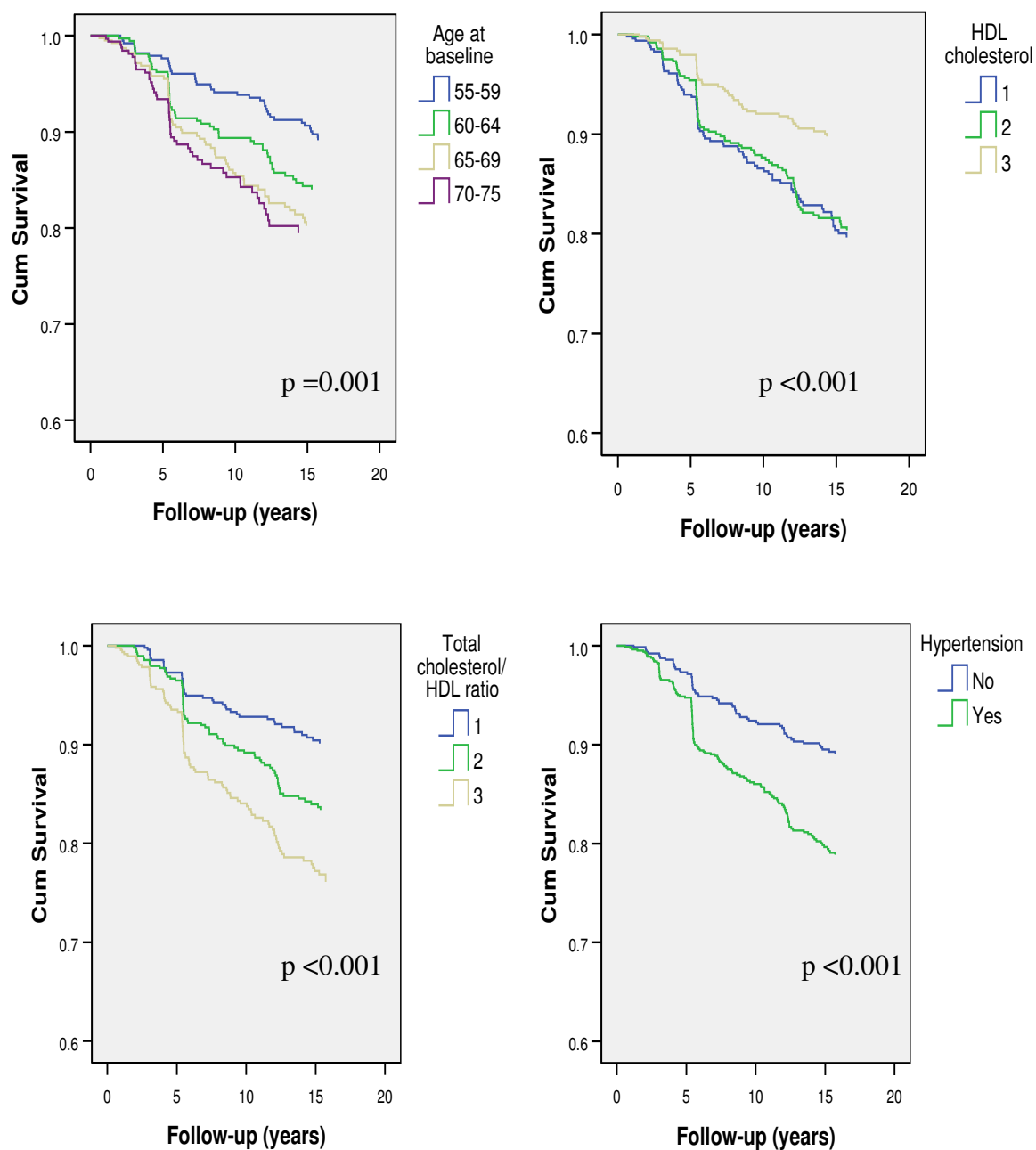
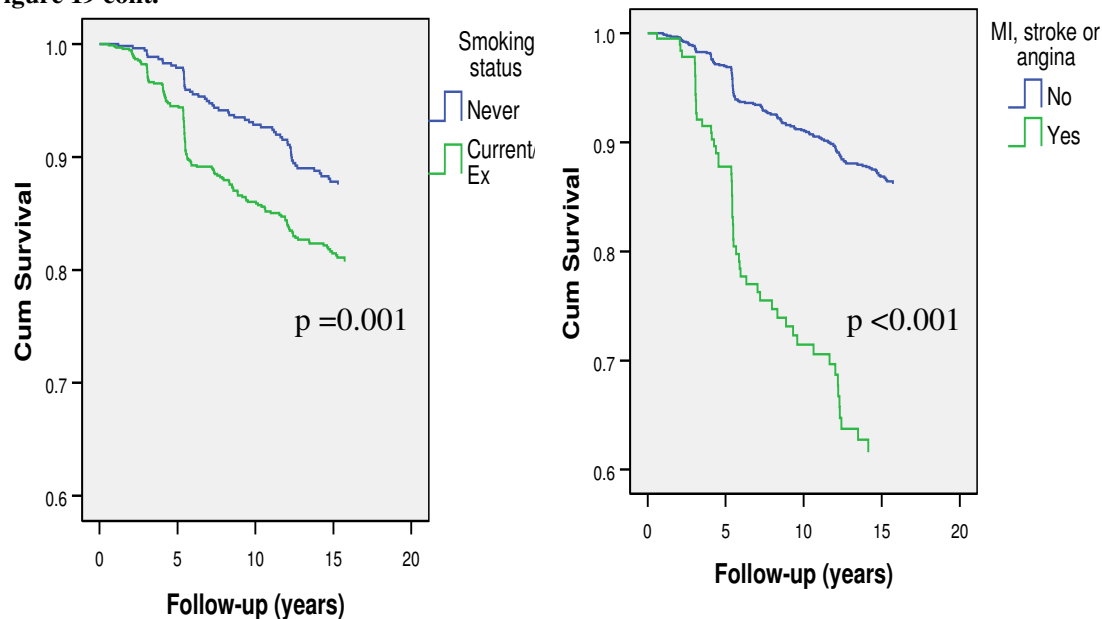


Figure 19 cont.



1: bottom tertile, 2: middle tertile, 3: top tertile.

MI: myocardial infarction.

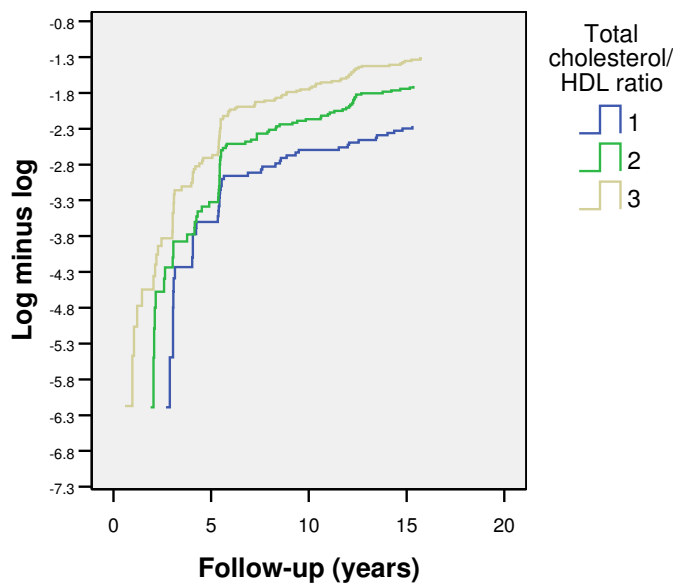
Cut-off points for age groups: 55-59, 60-64, 65-69, 70-75 years, for HDL cholesterol: 1.23, 1.59 mmol/L, for total cholesterol: 6.40, 7.49 mmol/L, total/ HDL cholesterol ratio: 4.32, 5.60. Never smokers: 930 subjects, current/ ex smokers: 547 subjects, hypertensives: 815 subjects, non-hypertensive: 704 subjects, MI, stroke or angina at baseline: 204 subjects, no cardiovascular disease at baseline: 1,315 subjects.

Table 40 Hazard ratios (95% CI) of cardiovascular risk factors for the development of peripheral arterial disease (PAD)

	Hazard ratio (95% CI) for development of PAD			
	Age, sex adjusted		Multivariable	
Total/ HDL cholesterol (top vs. bottom tertile)	2.54	(1.75, 3.69)**	2.30	(1.57, 3.38)**
BMI (1SD increase)	1.02	(0.89, 1.17)	0.89	(0.77, 1.04)
Pack years of smoking (top vs. bottom tertile)	2.31	(1.65, 3.23)**	2.34	(1.67, 3.30)**
Diabetes (yes vs. no)	1.46	(0.86, 2.48)	0.99	(0.56, 1.74)
Hypertension (yes vs. no)	1.85	(1.37, 2.49)**	1.77	(1.30, 2.42)**
Leisure activity (no activity vs. strenuous)	1.67	(0.86, 3.28)	1.51	(0.75, 3.07)
MI, stroke or angina (yes vs. no)	3.16	(2.30, 4.32)**	2.37	(1.70, 3.31)**

** p value significant at 0.01 * p value significant at 0.05

Figure 20 Log-log survival plot for incident peripheral arterial disease (PAD) per tertiles of total/ HDL cholesterol



1: bottom tertile. 2 middle tertile, 3: top tertile
Cut-off points for total/ HDL cholesterol 6.40 and 7.49 mmol/L

6.4 Inflammatory markers and incident PAD

In this part, the associations between inflammatory markers and incident PAD were examined. Throughout this analysis only the 1,519 subjects without baseline PAD were included.

6.4.1 Descriptive analysis

Inflammatory markers, CRP, IL-6 and ICAM-1 but not VCAM-1 and E-selectin were significantly elevated at baseline in those subjects who experienced symptomatic PAD during the follow up period (Table 41). When the PAD events were further categorized in moderate PAD and severe PAD, only ICAM-1 had a significant response dose relationship with disease severity (Figure 21). Median (interquartile range) ICAM-1 was 235 (201, 275) and 248 (206, 290) in subjects who developed modest and severe PAD respectively compared to 213 (184, 254) for the healthy group. On the contrary, subjects who experience moderate PAD and severe PAD had median IL-6 (interquartile range) 2.58 pg/mL (1.77, 3.93) and 2.51 pg/mL (1.68, 3.52) compared to 2.08 pg/mL (1.36, 3.41) in the healthy group (Figure 21).

6.4.2 Univariable associations with incident PAD

Survival curves for PAD development per tertile of each inflammatory marker are shown in Figure 22 along with the corresponding p value for the log rank test. The survival curves along with the log rank tests showed that top tertiles of CRP, IL-6 and ICAM-1 were associated with increased risk of developing PAD. The crude hazard ratio (95% CI) for people in the top tertile of these markers was 2.02 (1.33,

3.08) for CRP, 2.49 (1.61, 3.85) for IL-6 and 2.87 (1.92, 4.30) for ICAM-1. Figure 22 shows tertiles of VCAM-1 and E-selectin which did not differ in their risk of PAD development as their survival curves did not seem to separate over time.

6.4.3 Multivariable associations with incident PAD

Hazard ratios (95% CI) for PAD between top and bottom tertile of each marker and with various degrees of adjustments are shown for all inflammatory markers in Table 42. CRP and IL-6 showed significant associations in analysis adjusted for age and sex and both predicted a 2-fold or greater increase in the risk of having a PAD event in subjects falling in the top tertile of their distribution compared to those in the bottom tertile. However, their hazard ratios were gradually attenuated by adjustments for cardiovascular risk factors and then for CVD at baseline. Therefore, at the final multivariable analysis their hazard ratios lost statistical significance. For example, the hazard ratio (95% CI) of IL-6 was reduced from 2.14 (1.37, 3.35) to 1.48 (0.93, 2.35) after adjustments for risk factors and baseline CVD (top vs. bottom tertile). Likewise, the hazard ratio (95% CI) of CRP was reduced from 2.00 (1.30, 3.08) in the age and sex adjusted analysis to 1.46 (0.92, 2.32) in the multivariable analysis.

The considerable effect of cardiovascular risk factors on the association between either CRP or IL-6 with incident PAD was further investigated. Figure 23 shows the individual effect of each cardiovascular factor and of baseline CVD on the associations between PAD and IL-6 or CRP. Although none of these cardiovascular risk factors reduced the effect of the association below statistical significance, the effect of smoking and of baseline CVD was substantial and reduced the effect of the

associations in both CRP (hazard ratio: 1.69) and IL-6 (hazard ratio: 1.84). The ratio of total/ HDL cholesterol reduced the hazard ratio of CRP for PAD progression from 2.00 (age and sex adjusted analysis) to 1.72. Nevertheless, the hazard ratio of IL-6 was little changed after adjustment for total/HDL cholesterol ratio (from 2.14 to 2.09).

On contrast, ICAM-1 retained significant associations in all analyses and had hazard ratio (95% CI) between top and bottom tertile (1.78 (1.16, 2.73) $p=0.008$) in analysis adjusted for risk factors and CVD at baseline. Unlike for CRP and IL-6, the associations of ICAM-1 with PAD were little affected by baseline CVD adjustments and for example the age and sex adjusted hazard ratio (95% CI) for ICAM-1 was reduced from 2.54 (1.68, 3.84) to 2.49 (1.65, 3.74) after adjustments for baseline CVD. However, smoking attenuated considerably the associations between ICAM-1 and PAD reducing the hazard ratio (95% CI) to 2.17 (1.42, 3.32) (adjusted for age, sex and pack years of smoking). Likewise, ratio of total cholesterol reduced the hazard ratio (95% CI) to 2.35 (1.56, 3.56).

VCAM-1 and E-selectin did not show any significant associations with incident PAD in either the univariable or the multivariable analysis and their hazard ratios in age and sex adjusted analysis were 1.14 (0.78, 1.66) and 1.27 (0.86, 1.87) respectively. Finally, when all inflammatory variables were entered simultaneously in the model, ICAM-1 retained statistical significant associations and its hazard ratio (95 %CI) was 2.42 (1.39, 4.21) in age and sex adjusted analysis and 1.83 (1.02, 3.20) in the

multivariable analysis. Results for other inflammatory markers are shown in Table 42(b).

The assumption of proportional hazards was tested by stratifying the Cox regression by tertiles of each inflammatory marker and then plotting the log-log plot. All plots did not show serious violations of the proportional hazards assumption since the hazard ratios between different categories seemed constant over time. For example, the log-log plot of tertiles of IL-6 on Figure 24 shows that the hazard ratio between tertiles were approximately constant over time.

6.4.4 Sensitivity analysis

When the baseline population was limited to 1,315 subjects without baseline CVD (MI, stroke or angina) the aforementioned associations were not meaningfully changed but the CI were wider. In the age and sex adjusted analysis the hazard ratios (95% CI) between top and bottom tertile of CRP, IL-6 and ICAM-1 were 1.94 (1.18, 3.19), 2.24 (1.32, 3.80) and 1.72 (1.01, 2.94). These hazard ratios (95% CI) were reduced to 1.72 (1.01, 2.94), 1.69 (0.98, 2.92) and 1.41 (0.85, 2.33) for CRP, IL-6 and ICAM-1 in the analysis adjusted for cardiovascular risk factors.

Furthermore, when the baseline population was limited to those 1,273 subjects without subclinical atherosclerosis (defined by the ABI >0.9) the effect of the inflammatory markers was not very much changed. In the age and sex adjusted analysis the hazard ratios (95% CI) between top and bottom tertile of CRP, IL-6 and ICAM-1 were 1.79 (1.06, 3.03), 1.82 (1.08, 3.05) and 2.16 (1.33, 3.52). These hazard

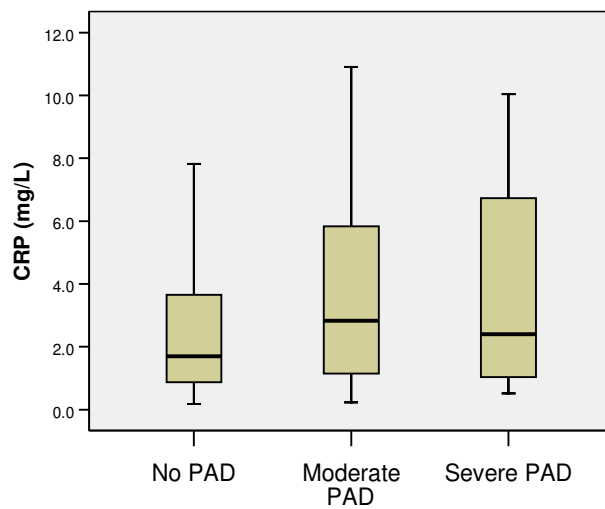
ratios (95% CI) were reduced to 1.37 (0.78, 2.41), 1.27 (0.74, 2.18) and 1.59 (0.95, 2.66) for CRP, IL-6 and ICAM-1 in the analysis adjusted for cardiovascular risk factors.

Table 41 Median (interquartile range) of inflammatory markers measured at baseline according to incident peripheral arterial disease (PAD)

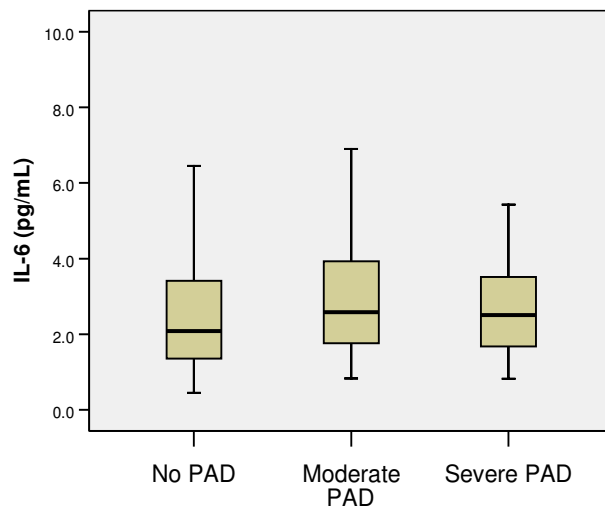
	N	Median (interquartile range)		p value
		Did not develop	Developed	
		PAD (N=1,310)	PAD (N=209)	
CRP (mg/L)	992	1.70 (0.87, 3.65)	2.81 (1.10, 6.02)	< 0.001
IL-6 (pg/mL)	1,038	2.08 (1.36, 3.41)	2.60 (1.77, 3.89)	0.003
ICAM-1 (ng/mL)	1,182	213 (184, 254)	235 (202, 277)	0.001
VCAM-1(ng/mL)	1,182	368 (324, 431)	381 (328, 441)	0.69
E-selectin (ng/mL)	1,179	41 (31, 52)	42 (33, 51)	0.64

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1.

Figure 21 Median (interquartile range) of C-reactive protein (CRP), interleukin-6 (IL-6), intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1) and E-selectin in people who remained free of peripheral arterial disease (PAD) and in those who developed moderate PAD and severe PAD after 17 years. The vertical bars denote those values of each marker that lie within 1.5 interquartile ranges of the limit of the interquartile range

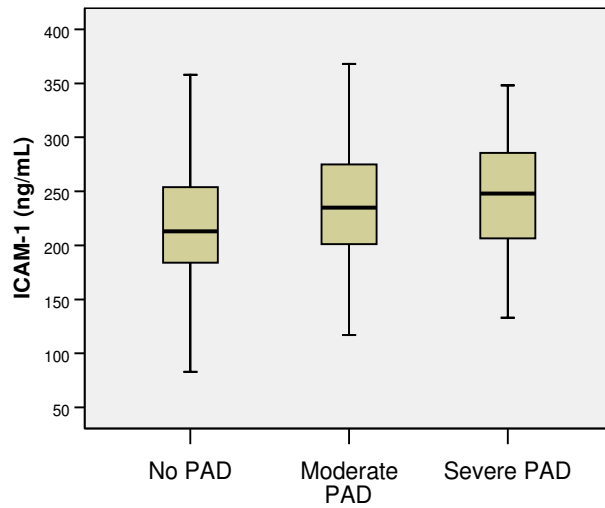


p for trend =0.16

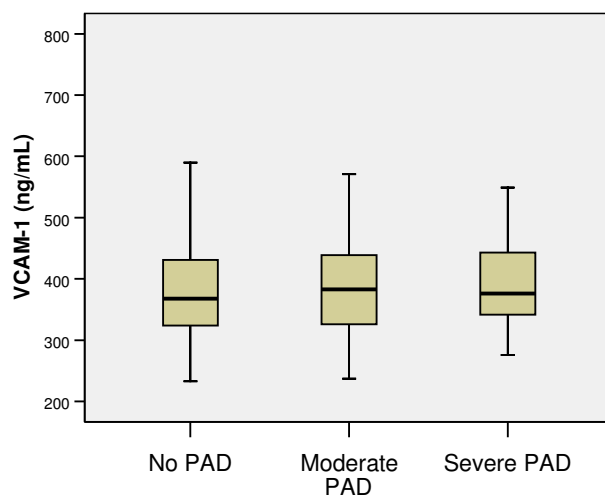


p for trend =0.28

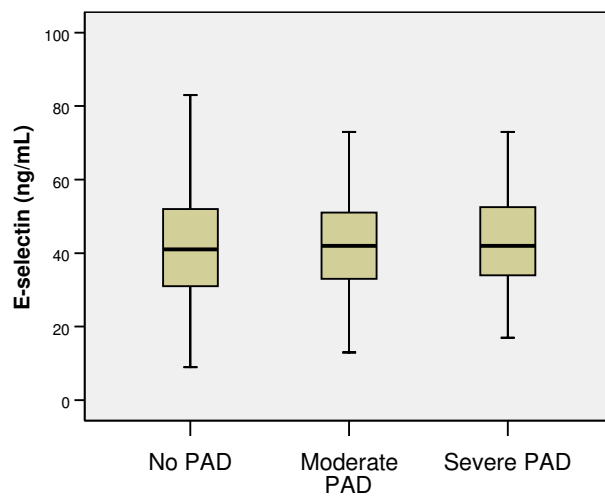
Figure 21 cont.



p for trend =0.001



p for trend =0.41



p for trend =0.49

Figure 22 Survival curves for incident peripheral arterial disease (PAD) per tertiles of inflammatory markers, p values correspond to the log rank test

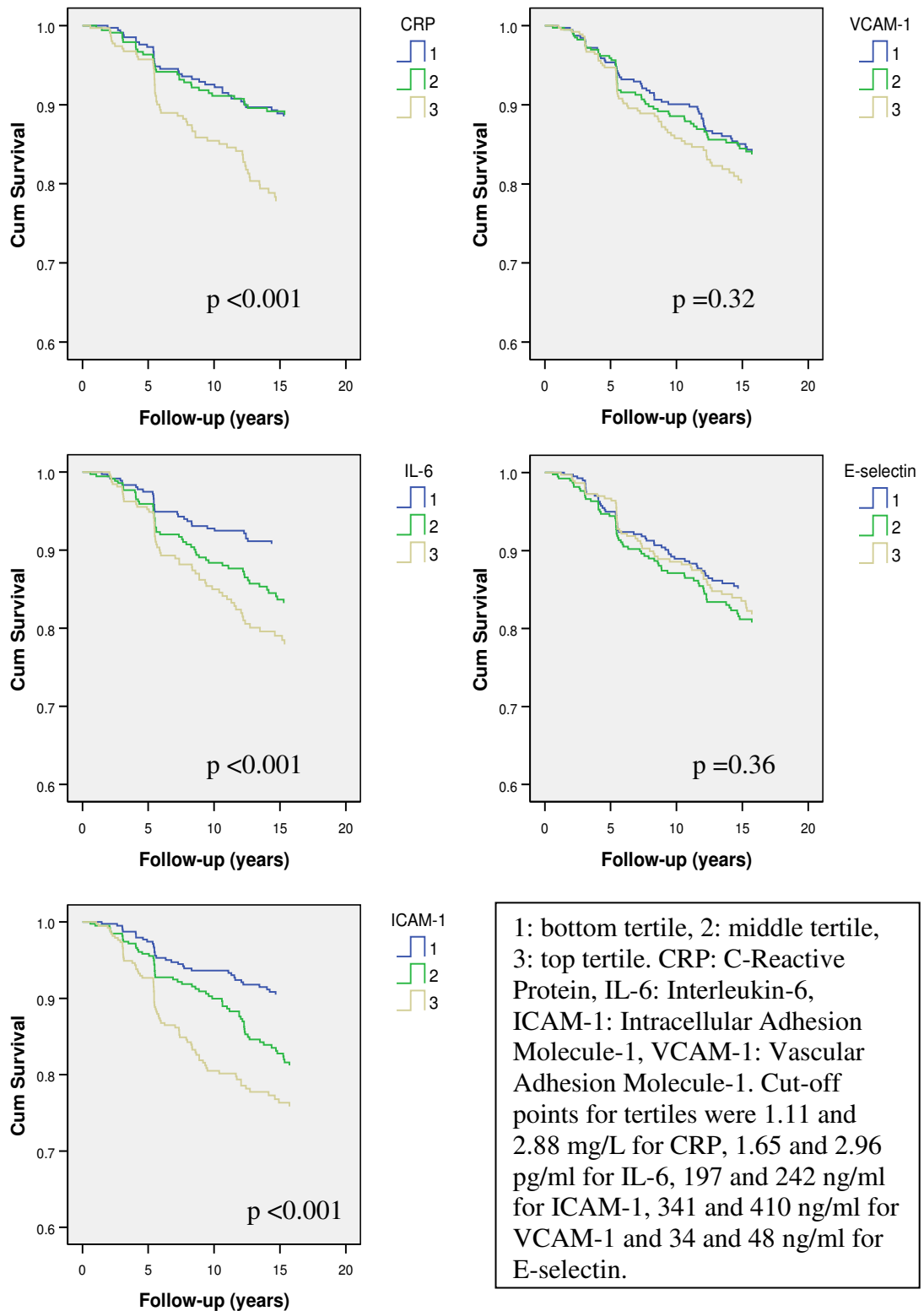


Table 42 Hazard ratios (95% CI) for development of peripheral arterial disease (PAD)

Hazard ratio for PAD when each marker is entered independently in the						
(a)	model adjusted for:					
	+ age, sex		+ risk factors [†]		+ cardiovascular disease [‡]	
CRP	2.00	(1.30, 3.08)**	1.62	(1.02, 2.56)*	1.46	(0.92, 2.32)
IL-6	2.14	(1.37, 3.35)**	1.63	(1.03, 2.60)*	1.48	(0.93, 2.35)
ICAM-1	2.54	(1.68, 3.84)**	1.73	(1.12, 2.67)**	1.78	(1.16, 2.73)**
VCAM-1	1.14	(0.78, 1.66)	1.01	(0.69, 1.48)	0.99	(0.68, 1.46)
E-selectin	1.27	(0.86, 1.87)	1.27	(0.86, 1.87)	1.10	(0.74, 1.64)
Hazard ratio for PAD when all markers are entered simultaneously in the						
(b)	model adjusted for:					
	+ age, sex		+ risk factors [†]		+ cardiovascular disease [‡]	
CRP	1.38	(0.82, 2.34)	1.43	(0.81, 2.50)	1.32	(0.75, 2.33)
IL-6	1.46	(0.83, 2.56)	1.34	(0.76, 2.37)	1.28	(0.72, 2.27)
ICAM-1	2.42	(1.39, 4.21)**	1.77	(0.98, 3.19)	1.83	(1.02, 3.30)*
VCAM-1	0.92	(0.56, 1.52)	0.86	(0.51, 1.43)	0.83	(0.50, 1.39)
E-selectin	0.64	(0.37, 1.08)	0.61	(0.35, 1.08)	0.60	(0.34, 1.07)

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1.

[†] pack years smoking, total/ HDL cholesterol, BMI, diabetes, physical activity, hypertension

[‡] myocardial infarction, stroke, angina

**p <0.01, *p <0.05.

Figure 23 Hazard ratios (95% CI) for peripheral arterial disease (PAD) between top and bottom tertile of (a) C-reactive protein (CRP) and (b) interleukin-6 (IL-6) after adjustments for different cardiovascular risk factors

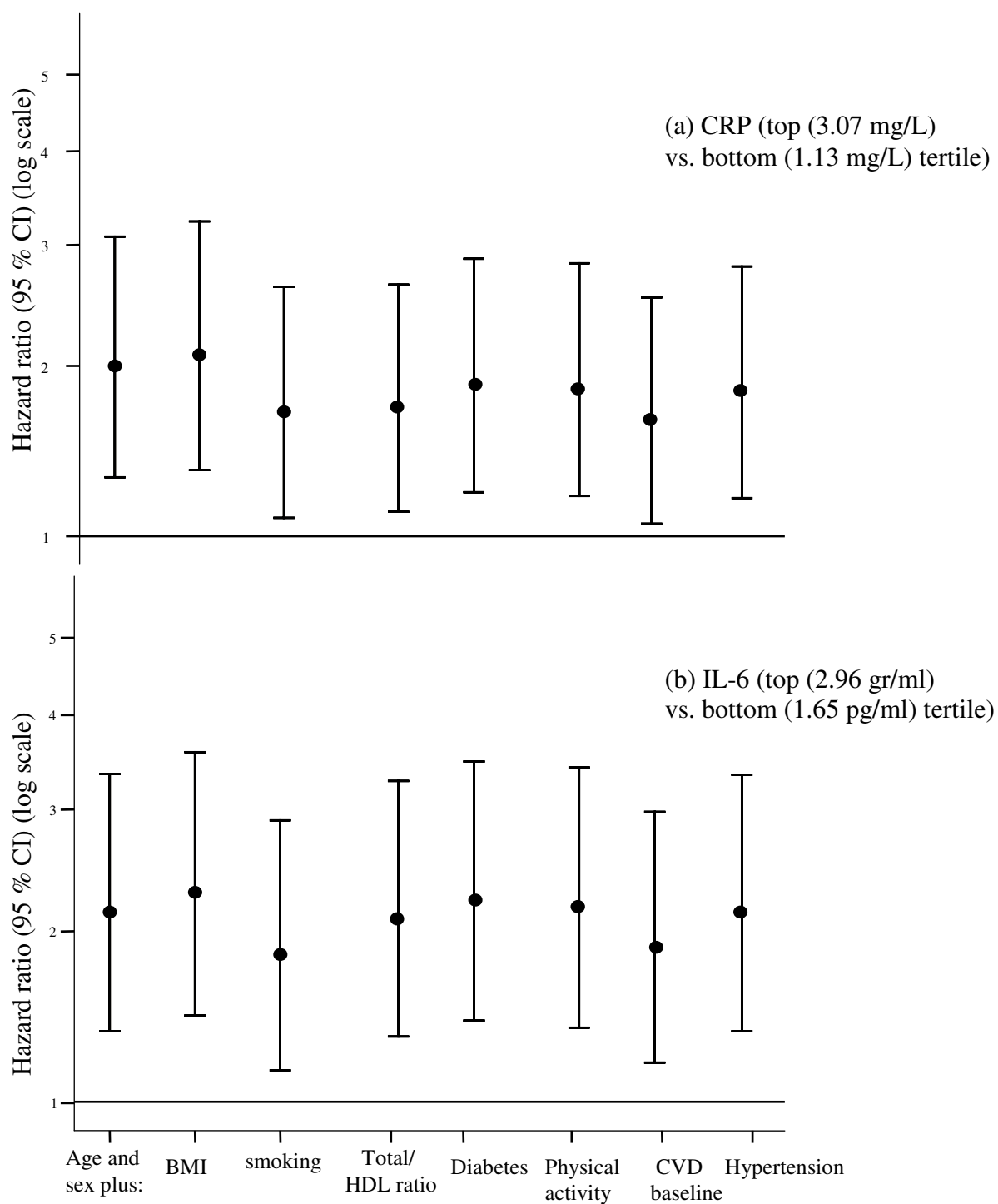
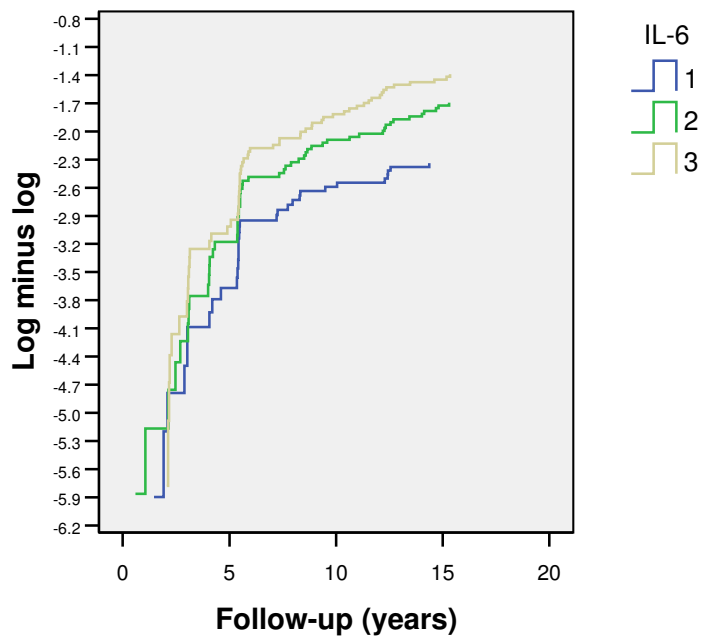


Figure 24 Log-log survival plot for incident peripheral arterial disease (PAD) for tertiles of interleukin-6 (IL-6)



1: bottom, 2: middle, 3: top tertile
Cut-off points for IL-6 tertiles: 1.65 and 2.96 pg/ml

6.5 Haemostatic markers and incident PAD

In this part, the association between haemostatic markers and incident PAD was examined. Throughout this analysis only the 1,519 subjects without baseline PAD were included in the analysis.

6.5.1 Descriptive analysis

Levels of fibrinogen, D-dimer and t-PA were higher in subjects who developed PAD than in those who did not develop PAD (Table 43). No significant elevations were found in levels of vWF, factor VII, FpA and F1+2 between subjects who developed and did not develop PAD as shown in Table 43. Figure 25 illustrates levels of haemostatic markers according to the severity of developed PAD. As this figure shows, D-dimer had a response dose relationship with disease severity with median (interquartile range) 90 (64, 133) and 97 (87, 140) in people who developed moderate and severe PAD respectively as opposed to 80 (57, 119) in those who did not develop PAD (p for trend =0.009). Plasma levels of t-PA showed similar results and were also higher with increasing disease severity (p for trend =0.02). Finally, as Figure 25 illustrates, other haemostatic markers failed to show a significant dose response relationship with disease severity.

6.5.2 Univariable associations with incident PAD

Survival curves for PAD development per tertile of each haemostatic marker are shown in Figure 26 along with the corresponding p value for the log rank test. Fibrinogen, D-dimer and t-PA had significant associations with PAD development.

Their crude hazard ratio (95% CI) between top and bottom tertile was 2.82 (1.99, 4.06) for fibrinogen, 2.39 (1.66, 3.44) for D-dimer and 2.23 (1.55, 3.20) for t-PA. As Figure 26 illustrates, small differences across tertiles of either vWF or factor VII were observed which were not statistically significant.

6.5.3 Multivariable associations with incident PAD

Hazard ratios (95% CI) for PAD between top and bottom tertile of each marker are shown for all haemostatic markers in Table 44. Fibrinogen and D-dimer showed significant associations in analysis adjusted for age and sex and both predicted a 2 fold or greater increase in the risk of having a PAD event in subjects falling in the top tertile of their distribution compared to those falling in the bottom tertile.

However, their hazard ratios were gradually decreased by adjustments for cardiovascular risk factors and then for CVD at baseline. Nevertheless, they did not lose statistical significance and at the multivariable adjusted analysis their hazard ratios (95% CI) was 2.18 (1.48, 3.23) for fibrinogen and 1.73 (1.16, 2.57) for D-dimer (top vs. bottom tertile).

Levels of t-PA were also significantly associated with incident PAD in age and sex adjusted analysis. However, its hazard ratio (95% CI) was attenuated after adjustments for CVD risk factors and baseline CVD from 1.80 (1.23, 2.62) to 1.26 (0.84, 1.89) (top vs. bottom tertile). Levels of vWF, factor VII, FpA, F1+2 did not show any significant associations with incident PAD in either the univariable or the multivariable analysis. Finally, when all inflammatory variables were entered simultaneously in the model, none retained statistical significant associations in age

and sex adjusted analysis or the cardiovascular risk factors adjusted analysis. Results for this analysis are shown in Table 44(b).

The assumption of proportional hazards was tested by stratifying the Cox regression by tertiles of each haemostatic marker and then plotting the log-log plot. All plots did not show serious violations of the proportional hazards assumption since the hazard ratios between different categories seemed constant over time. For example, the log-log plot of tertiles of fibrinogen on Figure 27 shows that the hazard ratio between tertiles was approximately constant over time although the instantaneous hazards varied.

6.5.4 Sensitivity analysis

When the baseline population was limited to 1,315 subjects without baseline CVD (MI, stroke or angina) the aforementioned associations were not meaningfully changed but the CI were wider. For example, in the age and sex adjusted analysis the hazard ratios (95% CI) between top and bottom tertile of fibrinogen, D-dimer and t-PA were 2.80 (1.37, 3.23), 2.11 (1.37, 3.23) and 1.90 (1.24, 2.91). These hazard ratios (95% CI) were reduced to 2.25 (1.37, 3.69), 2.58 (1.49, 4.47) and 1.45 (0.82, 2.54) for fibrinogen, D-dimer and t-PA in the analysis adjusted for cardiovascular risk factors.

Furthermore, when the baseline population was limited to those 1,273 subjects without subclinical atherosclerosis (defined by the ABI >0.9) the effect of the haemostatic markers was little changed. In the risk factors adjusted analysis the

hazard ratios (95% CI) between top and bottom tertile of fibrinogen and D-dimer were higher than the corresponding hazard ratios of the total population. In detail the hazard ratios (95% CI) for fibrinogen and D-dimer were 3.08 (1.78, 5.54) and 2.73 (1.51, 4.92).

Table 43 Median (interquartile range) of haemostatic markers measured at baseline according to incident peripheral arterial disease (PAD)

	N	Median (Interquartile range)		p value
		Did not develop PAD (N=1,310)	Developed PAD (N=209)	for difference
Fibrinogen (g/L)	1,548	2.6 (2.2, 3.03)	2.8 (2.4, 3.3)	< 0.001
D-dimer (ng/mL)	1,436	80 (57, 119)	94 (68, 134)	0.03
t-PA (ng/mL)	1,362	7.0 (5.0, 9.5)	8.2 (6.1, 10.5)	< 0.001
vWF (IU/dl)	1,446	106 (80, 139)	110 (84, 139)	0.58
Factor VII (IU/dl)	1,031	90 (70, 115)	65 (70, 125)	0.32
F1+2 (nmol/L)	1,062	1.7 (1.3, 2.1)	1.7 (1.4, 2.1)	0.88
FPA (ng/mL)	1,580	1.4 (1.0, 2.0)	1.5 (1.0, 2.0)	0.37

t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

Figure 25 Median (interquartile range) of fibrinogen, D-dimer, tissue plasminogen activator (t-PA), von Willebrand factor (vWF), factor VII, fibrinopeptide A (FpA) and prothrombin fragments 1+2 (F1+2) in people who remained free of peripheral arterial disease (PAD) and in those who developed moderate PAD and severe PAD after 17 years. The vertical bars denote those values of each marker that lie within 1.5 interquartile ranges of the limit of the interquartile range. P values correspond to p for trend calculated with ANOVA

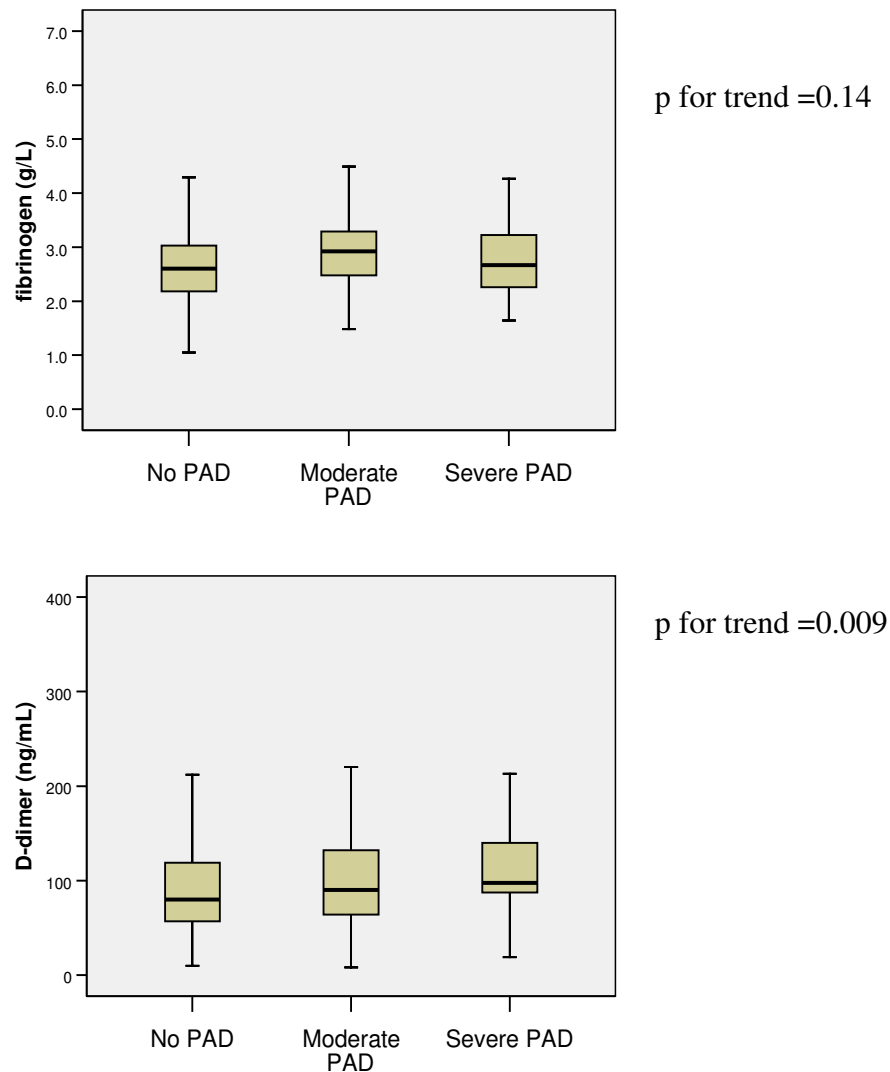


Figure 25 cont.

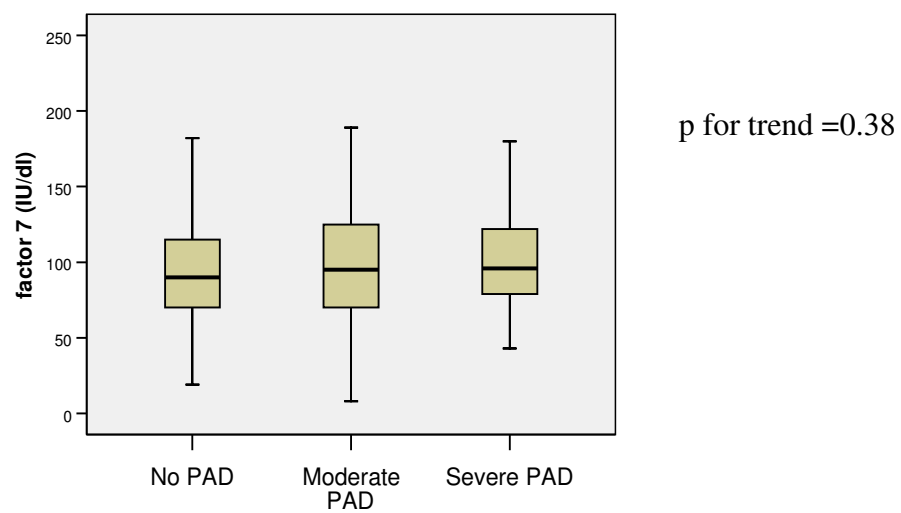
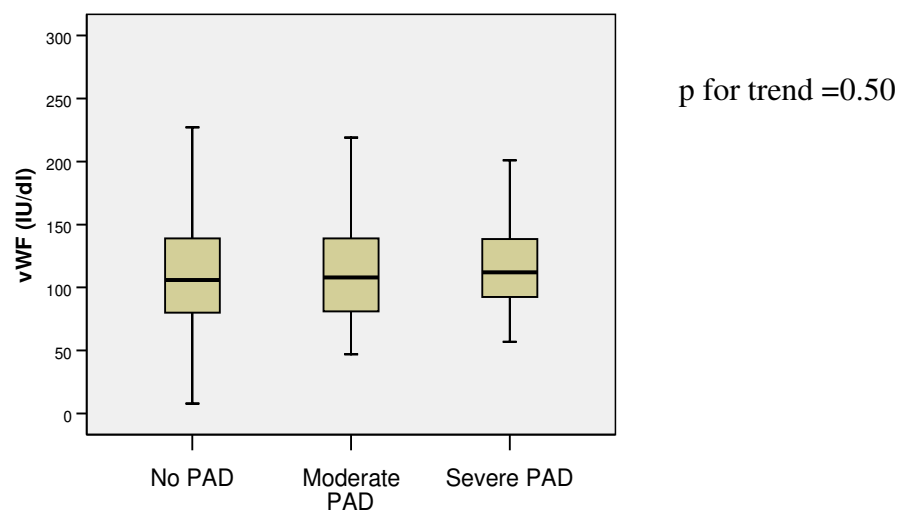
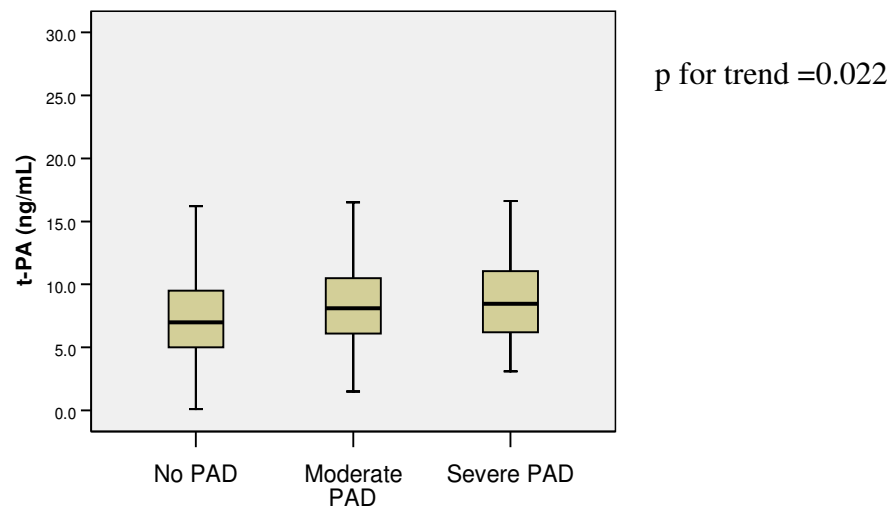


Figure 25 cont.

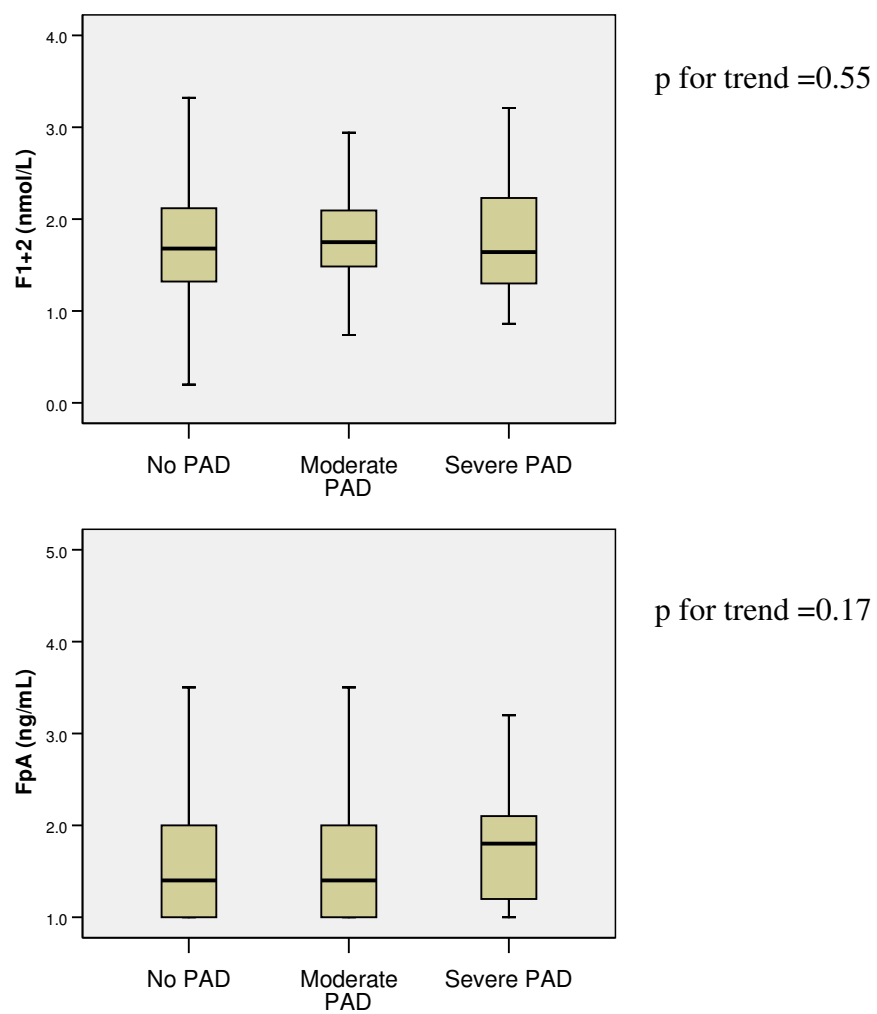


Figure 26 Survival curves for incident peripheral arterial disease (PAD) per tertiles of haemostatic markers, p values correspond to the log rank test

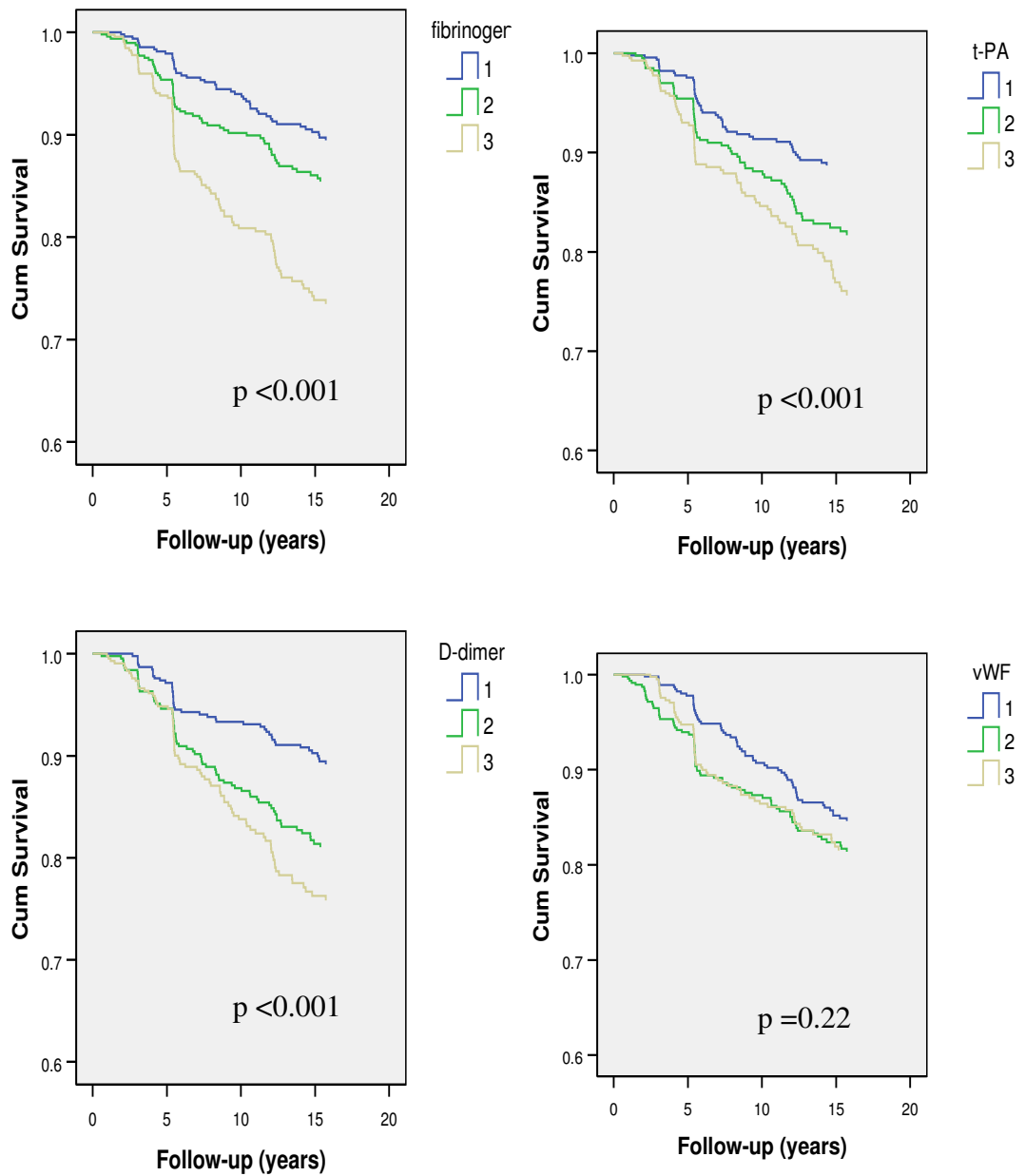


Figure 26 cont.

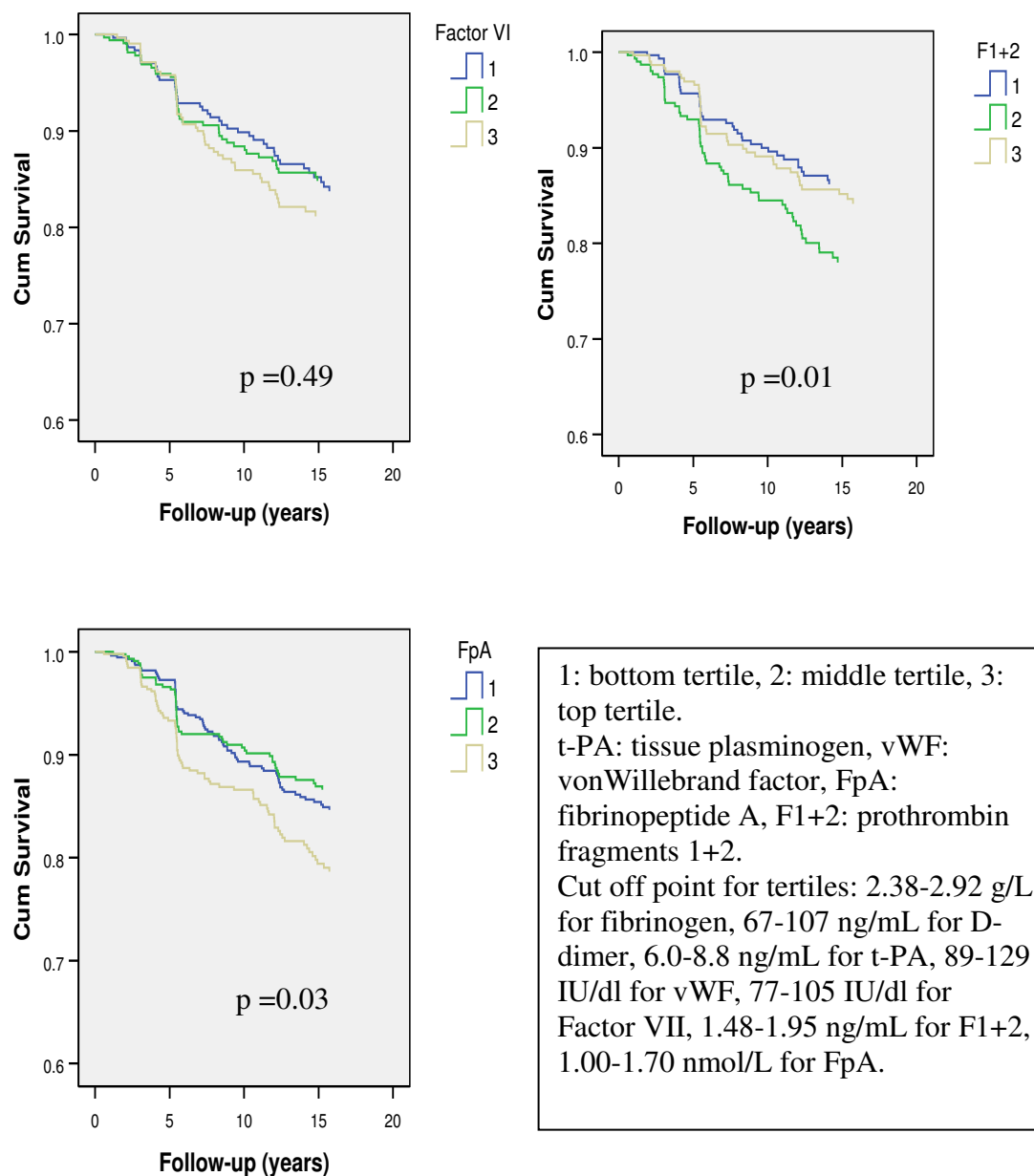


Table 44 Hazard ratios (95% CI) for development of peripheral arterial disease (PAD) between top and bottom tertile of each haemostatic marker

Hazard ratio (95% CI) for PAD when each marker is entered						
(a) independently in the model adjusted for:						
	+ age, sex		+ risk factors [†]		+ cardiovascular disease [‡]	
Fibrinogen	2.93	(2.03, 4.22)**	2.25	(1.54, 3.29)**	2.18	(1.48, 3.23)**
D-dimer	2.18	(1.48, 3.23)**	1.80	(1.21, 2.68)**	1.73	(1.16, 2.57)**
t-PA	1.80	(1.23, 2.62)**	1.30	(0.87, 1.95)	1.26	(0.84, 1.89)
VWF	1.17	(0.81, 1.67)	1.04	(0.73, 1.50)	1.01	(0.71, 1.45)
Factor VII	1.17	(0.77, 1.77)	1.20	(0.78, 1.83)	1.21	(0.79, 1.85)
F1+2	1.03	(0.65, 1.62)	1.03	(0.65, 1.63)	1.38	(0.99, 1.90)
FpA	1.35	(0.98, 1.86)	1.39	(1.00, 1.92)	1.03	(0.65, 1.62)
(b) Hazard ratio for PAD when all markers are entered simultaneously in the model adjusted for:						
	+ age, sex		+ risk factors [†]		+ cardiovascular disease [‡]	
Fibrinogen	1.48	(0.77, 2.82)	1.38	(0.72, 2.64)	1.31	(0.68, 2.52)
D-dimer	1.95	(0.97, 3.96)	1.56	(0.75, 3.23)	1.65	(0.79, 3.43)
t-PA	1.15	(0.64, 2.09)	0.95	(0.50, 1.81)	0.99	(0.52, 1.88)
vWF	0.76	(0.40, 1.46)	0.64	(0.33, 1.24)	0.69	(0.35, 1.33)
Factor VII	0.99	(0.55, 1.80)	1.16	(0.62, 2.18)	1.15	(0.62, 2.17)
F1+2	1.03	(0.57, 1.88)	1.09	(0.58, 2.04)	1.12	(0.67, 2.34)
FpA	1.64	(0.94, 2.86)	1.66	(0.94, 2.92)	1.73	(0.99, 3.04)

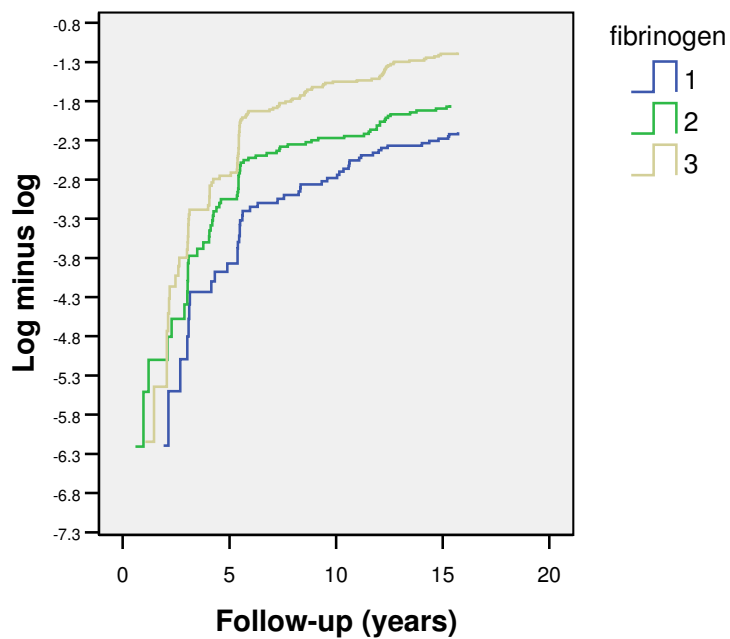
t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

[†] pack years smoking, diabetes, BMI, total/ HDL cholesterol ratio, physical activity and hypertension.

[‡] myocardial infarction, stroke, angina.

** $p \leq 0.01$ and * $p \leq 0.05$.

Figure 27 Log – log plot for incident peripheral arterial disease (PAD) per tertiles of fibrinogen



1:top, 2:middle, 3:top tertile
Cut-off points for fibrinogen: 2.38, 2.92 g/L

6.6 Additive effect of inflammatory and haemostatic markers on incident PAD

In this part, the additive effect between inflammatory and haemostatic markers on their associations with incident PAD was investigated.

6.6.1 Independent and joint effect

The previous analyses showed that the inflammatory marker ICAM-1 and the haemostatic markers fibrinogen and D-dimer were significant and independent predictors of PAD development after 17 years of follow up period. A stepwise Cox regression model was also conducted. All cardiovascular risk factors and history of CVD were forced into the model whereas inflammatory and haemostatic variables that had significant hazard ratios in the univariable analysis were selected using backward selection process. In the final step, only ICAM-1 and D-dimer were included in the model.

The additive effect of ICAM-1 and D-dimer along with fibrinogen, which had the greatest single independent effect with incident PAD, was further investigated. When fibrinogen was adjusted for D-dimer, its effect was attenuated and had borderline significance at the multivariable analysis (Table 45). At the same model D-dimer remained statistical significant with hazard ratio (95% CI) 1.94 (1.15, 3.27) (top vs. bottom tertile). Similarly, D-dimer retained significant associations when adjusted for ICAM-1 levels and its hazard ratio (95% CI) was 2.35 (1.35, 4.10) (top vs. bottom tertile) in the multivariable analysis.

Moreover, D-dimer was an independent predictor of PAD when all 3 markers, fibrinogen, D-dimer and ICAM-1, were entered simultaneously into the Cox regression model with hazard ratio (95% CI) 1.87 (1.05, 3.35) (top vs. bottom tertile) in the multivariable analysis. The same was not true for fibrinogen and ICAM-1, which had weaker association in this model and failed to reach statistical significance in the adjusted analysis (Table 33).

6.6.2 Combined variables: ICAM-1, fibrinogen and D-dimer

The combined effect of fibrinogen, D-dimer and ICAM-1 was investigated further. The population at baseline was divided into 4 mutually exclusive groups according to their baseline levels of D-dimer and fibrinogen: levels of fibrinogen and D-dimer in the top tertile (219 subjects), levels of fibrinogen in the top tertile and levels of D-dimer in the middle or bottom tertile (211 subjects), levels of D-Dimer in the top tertile and levels of fibrinogen in the middle or bottom tertile (223 subjects) and finally levels of both D-dimer and fibrinogen in the middle or bottom tertile (696 subjects). Compared to the reference group of people with both variables at the bottom or middle tertile, people with only D-dimer elevated had hazard ratio (95% CI) 1.58 (0.92, 2.73), people with only fibrinogen elevated had hazard ratio (95% CI) 2.35 (1.47, 3.77) and people with both variables elevated had hazard ratio (95% CI) 2.62 (1.64, 4.19) in analysis adjusted for CVD risk factors and baseline CVD.

The baseline population was also divided according to fibrinogen and ICAM-1 levels and according to D-dimer and ICAM-1 levels into 4 groups respectively but here a linear trend between increasing number of elevated variables was less apparent.

People falling in the top tertile of fibrinogen and ICAM-1 had hazard ratio (95% CI) 2.69 (1.63, 4.46) compared to those in the bottom or middle tertile of both variables. Similarly, people falling in the top tertile of both D-dimer and ICAM-1 had hazard ratio (95% CI) 2.18 (1.24, 3.82) compared to those in the bottom or middle tertile of both variables, in the multivariable analysis.

6.6.3 Combined variables: IL-6 and D-dimer

Moreover, analysis on inflammatory and haemostatic markers and the progression of peripheral atherosclerosis showed an additive effect of D-dimer and IL-6 on ABI decline. This effect was tested in relation to PAD development also. Again, individuals were divided into 4 mutually exclusive groups according to their baseline levels of D-dimer and IL-6: levels of IL-6 and D-dimer in the top tertile (171 subjects), levels of IL-6 in the top tertile and levels of D-dimer in the middle or bottom tertile (180 subjects), levels of D-Dimer in the top tertile and levels of IL-6 in the middle or bottom tertile (170 subjects) and finally levels of both D-dimer and IL-6 in the middle or bottom tertile (506 subjects). However, there was not a clear trend across groups and IL-6 did not seem to add to the effect of D-dimer on PAD prediction. More specifically, in age and sex adjusted analysis, the hazard ratio for people with elevated D-dimer only was 2.41 (1.34, 4.34), with elevated IL-6 only was 1.81 (0.99, 3.28) and with elevated both variables was 2.40 (1.35, 4.25).

Table 45 Hazard ratios (95% CI) for development of peripheral arterial disease (PAD) between top and bottom tertile of ICAM-1, fibrinogen and D-dimer

Hazard ratio for PAD when each marker is entered independently in the model adjusted for:			
	+ age, sex	+ risk factors [†]	+ cardiovascular disease [‡]
Fibrinogen and ICAM-1 entered simultaneously			
Fibrinogen	2.10 (1.26, 3.51)**	2.04 (1.21, 3.42)**	1.94 (1.15, 3.27)*
ICAM-1	2.34 (1.32, 4.15)**	1.68 (0.93, 3.02)	1.80 (0.99, 3.25)
Fibrinogen and D-dimer entered simultaneously			
Fibrinogen	1.88 (1.19, 2.98)**	1.75 (1.09, 2.80)*	1.80 (0.99, 3.25)
D-dimer	2.40 (1.44, 3.99)**	1.98 (1.18, 3.34)*	1.94 (1.15, 3.27)*
ICAM-1 and D-dimer entered simultaneously			
ICAM-1	2.36 (1.36, 4.08)**	1.65 (0.93, 2.92)	1.77 (1.00, 3.15)
D-dimer	2.83 (1.64, 4.89)**	2.50 (1.44, 4.35)**	2.35 (1.35, 4.10)**
ICAM-1, fibrinogen and D-dimer entered simultaneously			
ICAM-1	2.17 (1.22, 3.86)**	1.58 (0.87, 2.85)	1.70 (0.94, 3.09)
Fibrinogen	1.70 (1.00, 2.89)*	1.76 (1.03, 3.01)*	1.71 (0.99, 2.93)
D-dimer	2.27 (1.29, 4.01)**	1.98 (1.11, 3.52)**	1.87 (1.05, 3.35)*

ICAM-1: Intracellular Adhesion Molecule-1.

[†] pack years smoking, diabetes, BMI, total/ HDL cholesterol ratio, physical activity and hypertension.

[‡] myocardial infarction, stroke, angina.

** p <0.01, *p<0.05.

6.7 Fibrinogen, smoking, cholesterol levels and incident PAD

Fibrinogen had the strongest association with future PAD in both age and sex and the multivariable analysis among the other markers studied here. In addition, fibrinogen along with cholesterol and smoking were the only markers that predicted a 2 fold or greater increase in the risk of developing PAD compared to the other conventional and non-conventional markers studied here (top vs. bottom tertile). In detail, the hazard ratio (95% CI) for the top vs. the bottom tertile of fibrinogen was 2.18 (1.48, 3.23) ($p < 0.001$), of total/HDL cholesterol was 2.05 (1.39, 3.02) ($p < 0.001$) and of smoking was 2.09 (1.47, 2.97) ($p < 0.001$) in the multivariable analysis. This relative importance of fibrinogen along with traditional risk factors smoking and cholesterol was investigated further.

6.7.1 Cholesterol levels

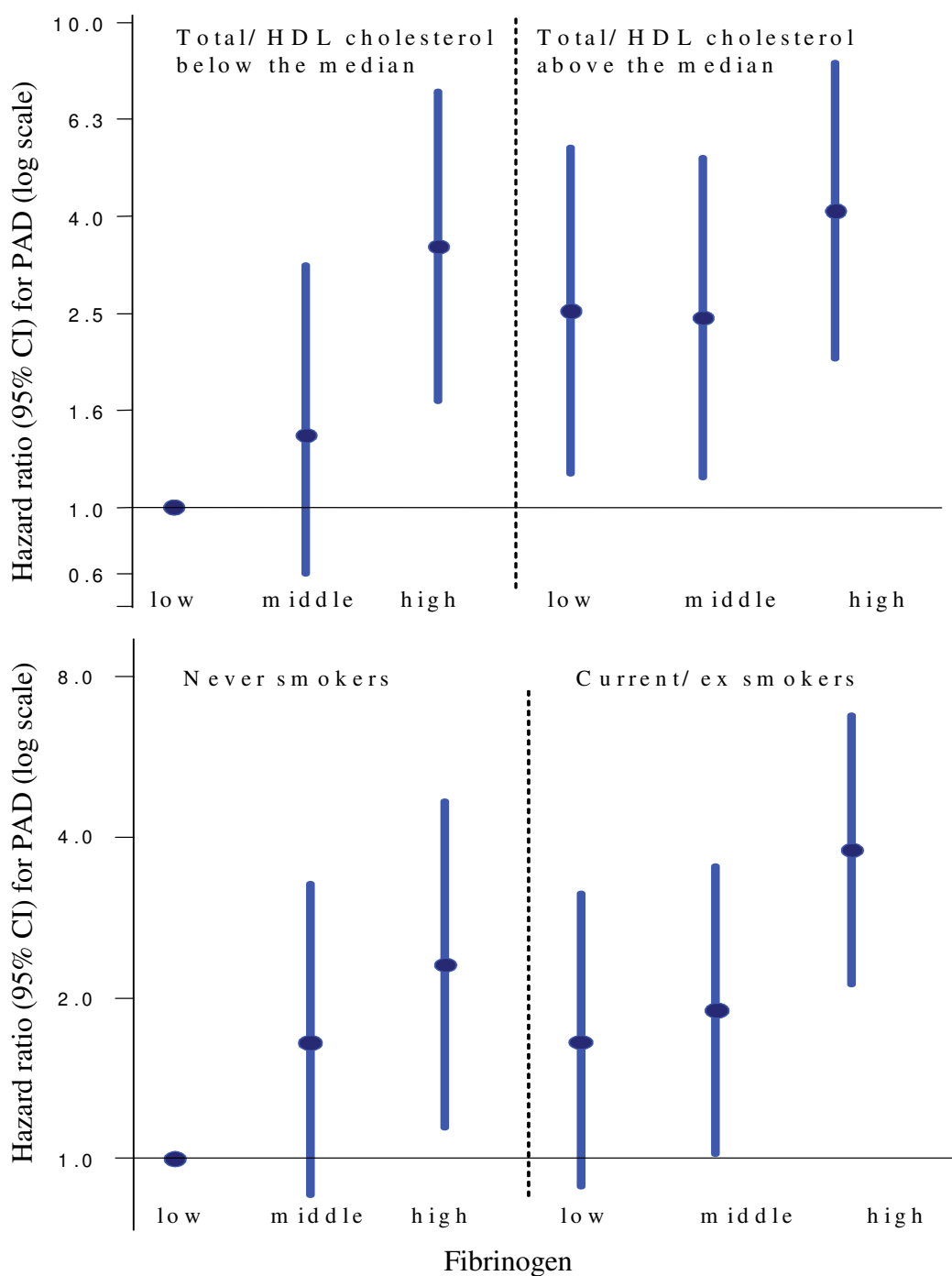
The baseline population was stratified according to total/ HDL cholesterol levels and fibrinogen into 6 groups: people with total/ HDL cholesterol ratio below the median and with fibrinogen at bottom, middle or top tertile (293, 266, 211 subjects respectively) and people with total/ HDL cholesterol ratio above the median with fibrinogen at bottom, middle or top tertile (221, 248, 303 subjects respectively). Figure 28 shows the hazard ratio for each group compared to the group of people with total/ HDL cholesterol below the median and fibrinogen levels at the bottom tertile (reference group). People with increased fibrinogen levels (top tertile) had significant associations with PAD development in the multivariable analysis whether

their total/ HDL cholesterol ratio was below or above the median. In fact, the hazard ratio (95% CI) was 3.46 (1.66, 7.21) ($p=0.001$) for people with fibrinogen at the top tertile and total/ HDL cholesterol ratio below the median and 2.49 (1.16, 5.32) ($p=0.02$) for people with fibrinogen at the top tertile and total/ HDL cholesterol ratio above the median compared to the reference group.

6.7.2 Smoking

Moreover, the baseline population was divided into 6 groups according to smoking status and fibrinogen levels: never smokers with fibrinogen levels at top, bottom or top tertile (218, 191, 141 subjects respectively) and ever (present and ex) smokers with fibrinogen levels at top, middle or bottom tertile (281, 310, 364 subjects respectively). As shown in Figure 28, people with increased fibrinogen levels were at increased risk of developing PAD whether they were smokers (hazard ratio (95% CI) 3.76 (2.11, 6.72)) ($p<0.001$) or never smokers (hazard ratio (95% CI) 2.30 (1.14, 4.63)) ($p=0.020$).

Figure 28 Hazard ratios (95% CI) for peripheral arterial disease (PAD) according to tertiles of fibrinogen and (a) total/ HDL cholesterol and (b) smoking status at baseline



The hazard ratios are adjusted for age, sex, BMI, diabetes, hypertension, physical activity, cardiovascular disease at baseline and (a) pack years smoking or (b) total/ HDL cholesterol. Reference group was the group of people with fibrinogen at the bottom tertile and (a) total/ HDL cholesterol below the median or (b) never smoker. Median for total/ HDL cholesterol: 4.96. Current/ ex smokers: 720 subjects, never smokers: 547 subjects. Cut-off point for tertiles of fibrinogen 1.38, 2.92 g/L.

6.8 Incident CVD

In this part the incidence of CVD, including myocardial infarction and stroke, in the baseline population over 17 years of follow up is described.

6.8.1 Incidence of myocardial infarction and stroke

After 17 years of follow up, 702 participants (44%) of the baseline population died. From those, 129 have died (18%) due to MI and 65 (9%) due to stroke. In addition, 146 subjects experienced at least one non fatal MI and 106 at least one stroke. From the 248 subjects that experience either fatal or no fatal MI, 33 (13%) subjects had two MIs recorded (fatal or non fatal) and 10 (4%) more than two MIs (fatal or non fatal). Also, from those 168 that experienced either fatal or non-fatal event of stroke, 24 (14%) experienced two events and 4 (2%) more than two stroke events. Moreover, 29 subjects (12% from those with MI and 17% from those with stroke) experienced both an event of MI and of stroke.

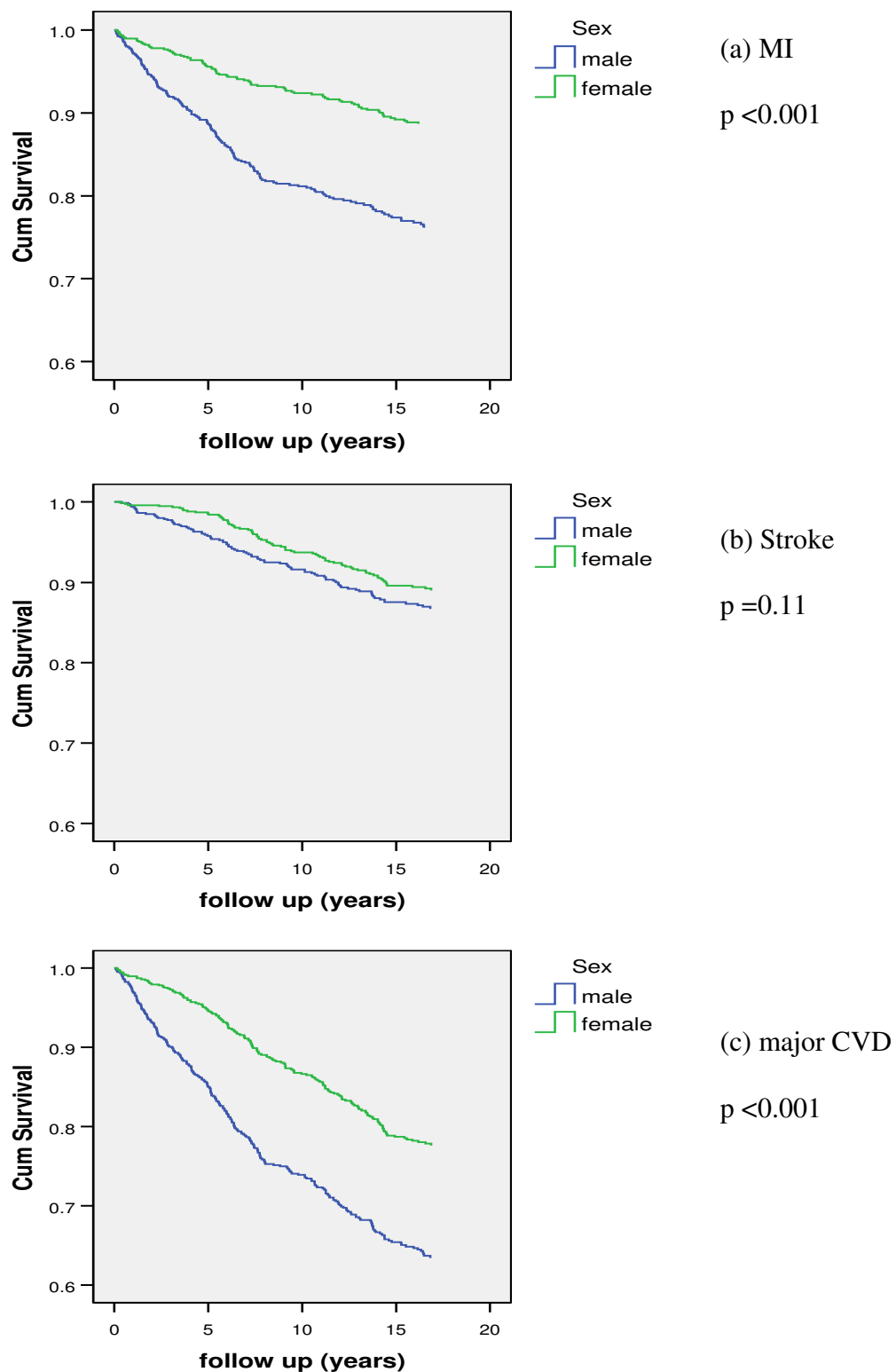
The mean time to diagnosis of incident MI for the study population was 135 months (range 3-198 months). The KM curve for MI and stroke for the males and females separately is shown in Figure 29. From the 248 events of MI, 168 (68%) have occurred in males and 80 (32%) in females (p for difference <0.001). The mean time to diagnosis for incident stroke was 91 months (range 4-203 months). Ninety two stroke events (55%) occurred in males and 76 (45%) in females (p for difference =0.11).

At baseline, 277 (17.4 %) subjects had a history of CVD defined as MI, stroke, angina or IC. Of them, 96 (34.6%) had an event of MI and 55 (19.9%) had an event of stroke during the follow up. On the other hand, from the 1,315 without clinical CVD at baseline, 152 (11.6 %) developed MI and 113 stroke (8.6 %).

6.8.2 Incidence of major CVD

During the 17 years of follow up, 35 (2.2%) subjects had had a CABG and 36 (2.3%) had had an angioplasty (64 or 4.0% had either of them). Of those 64, 31 (48.4%) had also been diagnosed with MI and 6 (9.4%) had also been diagnosed with stroke. A composite group of major CVD which included subjects who developed either MI, stroke or who had a CABG or an angioplasty was created. In total, 415 (26.1%) subjects developed at least one of those events during the follow up period. Of those events, 148 occurred in the 277 people with history of CVD at baseline (53.4% of them was diagnosed with major CVD during the follow up). The KM curve for major CVD for males and females separately is shown in Figure 29. In total, 258 (62%) major CVD events occurred in males and 157 (38%) in females (p for difference <0.001). The mean time to diagnosis was 82 months (range 1-202 months).

Figure 29 Survival curves for incident myocardial infarction (MI), stroke and major cardiovascular disease (CVD) over 17 years of follow up for males and females. P values correspond to log rank test



6.9 Cardiovascular risk factors and incident CVD

In this part, the association between traditional cardiovascular risk factors and incident major CVD, MI and stroke was examined.

6.9.1 Descriptive analysis

Table 46 lists the means (SD) of cardiovascular risk factors measured at baseline in those subjects who developed or did not develop major CVD. Subjects who developed major CVD were generally older and had higher percentage of males compared to those that did not develop PAD. Moreover, mean ratio of total/ HDL cholesterol and pack years of smoking were all significantly higher at baseline among subjects who developed major CVD compared to those who remained free of major CVD. On the contrary, no significant difference was seen for total cholesterol. Levels of HDL cholesterol were significantly lower in those who developed major CVD. Also, 9.0% suffered from diabetes at baseline among those who developed major CVD compared to 4.8% subjects with diabetes in the healthy group.

When the major CVD events were divided in MIs and strokes, total cholesterol levels and the ratio of total cholesterol was significantly higher in the MI group compared to the healthy group but not in the group of people who developed stroke. Finally, subjects with diabetes at baseline did not differ between those who developed stroke and who remained free of major CVD. On the other hand people with MI had increased percentage of subjects with diabetes (10.9 %) compared to the no major CVD group (5.0%) ($p < 0.001$).

6.9.2 Univariable and multivariable associations with incident CVD

Cox regression was used to calculate crude, age and sex adjusted and multi-adjusted hazard ratios (95% CI) for major CVD, MI or stroke and total/ HDL cholesterol, pack years of smoking, diabetes, BMI, physical activity, baseline CVD and ABI. Table 47 lists the multivariable analyses for major CVD and Table 48 for MI and stroke separately.

Hazard ratios for major CVD unadjusted and adjusted for age and sex were significant for all variables except for physical activity. In the multivariable analysis (all variables entered simultaneously in the model) total/ HDL cholesterol, pack years of smoking, baseline CVD and ABI were significant and independent predictors of future major CVD (Table 47). In particular, subjects with baseline history of CVD had more than a 2 fold increase in their risk for developing a major CVD (hazard ratio (95% CI) 2.58 (2.05, 3.25)). Similarly, those with total/ HDL cholesterol ratio or with pack-years of smoking in the top tertile of their distribution had hazard ratio (95% CI) 1.68 (1.27, 2.21) and 1.46 (1.13, 1.90), respectively. In addition, when smoking status (ever vs. never smokers) was fitted in the model instead of pack years of smoking, the results were almost the same and the HR (95% CI) for incident CVD in ever compared to never smokers was 1.39 (1.09, 1.77) (p=0.007).

When the events were divided in MI or stroke, hazard ratios were little changed. Total/ HDL cholesterol, pack years of smoking, baseline CVD and ABI were

significant and independent predictors of future MI (Table 48). Again, subjects with baseline history of CVD had more than a 2-fold increase in their risk for developing a major CVD (hazard ratio (95% CI) 2.80 (2.09, 3.74)). Also, those with total/ HDL cholesterol ratio or with pack-years of smoking in the top tertile of their distribution had hazard ratio (95% CI) 1.96 (1.35, 2.84) and 1.43 (1.01, 2.01), respectively. In addition, BMI, pack years of smoking, baseline history of CVD and ABI but not the ratio of total/ HDL cholesterol were independently associated with incident stroke (Table 48).

Proportional hazard assumption was examined for the all the variables entered in the Cox regression by stratifying the Cox regression with each covariate and by plotting the log-log survival plot. No serious deviations were observed. As an example, the log –log plot on Figure 30 shows that hazard ratios for major CVD between tertiles of total/ HDL cholesterol were constant over time.

6.9.3 Sensitivity analysis

When the analysis was limited to 1,315 subjects without history of CVD, results of Cox regression did not meaningfully change. In multivariable analysis, the hazard ratio (95% CI) for incident major CVD between top and bottom tertile of total/ HDL cholesterol was 1.87 (1.34, 2.61) and of pack years of smoking 1.80 (1.30, 2.49).

Also, per 1SD decrease of ABI the hazard ratio (95% CI) for future major CVD was 1.25 (1.09, 1.45) and per 1SD increase of BMI 1.12 (0.99, 1.26). Diabetes status and leisure activity did not show any significant associations with incident major CVD as in the previous analyses.

Table 46 Cardiovascular risk factors in subjects who did or did not developed major cardiovascular disease (CVD) over the 17 years of follow up

	Mean (SD)/ Percent		p value
	Did not develop CVD (N=1,177)	Developed CVD (N=415)	
Age (years)	64.4 (5.66)	66.2 (5.48)	< 0.001
Males	46.8 %	62.2 %	< 0.001
Smoking			
Never smokers	39.9 %	25.8 %	< 0.001
Ever smokers	60.1 %	74.2 %	
Pack years smoking ($\sqrt{}$)	2.7 (2.83)	3.6 (2.87)	< 0.001
BMI (kg/m ²)	25.5 (4.02)	26.0 (3.57)	0.01
Diabetes	4.8 %	9.0 %	0.002
Cholesterol/ HDL ratio	5.1 (2.04)	5.7 (1.67)	< 0.001
Total cholesterol (mmol/L)	7.0 (1.32)	7.1 (1.36)	0.13
HDL cholesterol (mmol/L)	1.5 (0.41)	1.3 (0.37)	< 0.001
Physical activity			
Light	36.9 %	38.2 %	
Moderate	42.7 %	41.3 %	0.55
Strength	10.0 %	11.8 %	
ABI	1.05 (0.16)	0.98 (0.22)	< 0.001
Cardiovascular disease			
Myocardial infarction	1.7 %	7.4 %	< 0.001
Stroke	1.3 %	4.0 %	0.003
Angina	5.0 %	19.4 %	< 0.001
IC	2.6 %	9.0 %	< 0.001
Any of the above	11.0 %	35.7 %	< 0.001

ABI: Ankle brachial index, IC: intermittent claudication.

Table 47 Hazard ratio (95% CI) for incident major cardiovascular disease (CVD) and baseline cardiovascular risk factors adjusted for all other factors in the model

Hazard ratio (95% CI) for development of major CVD		
Total/ HDL cholesterol (top vs. bottom tertile)	1.68	(1.27, 2.21)**
BMI (1SD increase)	1.06	(0.95, 1.17)
Pack years of smoking (top vs. bottom tertile)	1.46	(1.13, 1.90)**
Diabetes (yes vs. no)	0.74	(0.38, 1.43)
Leisure activity (no activity vs. strenuous)	1.51	(0.75, 3.07)
ABI (1SD decrease)	1.18	(1.06, 1.30)**
History of cardiovascular disease (yes vs. no)	2.58	(2.05, 3.25)**

ABI: Ankle brachial index

** p value <0.01

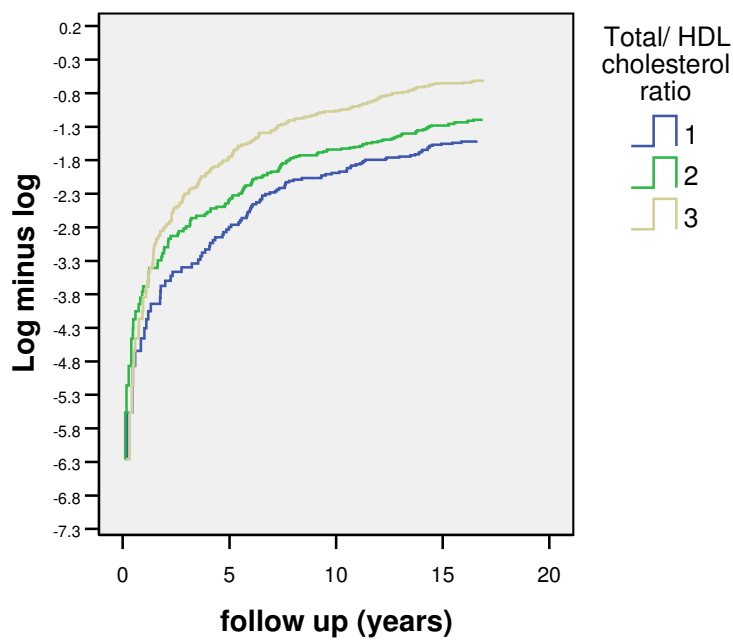
Table 48 Hazard ratio (95% CI) for incident myocardial infarction (MI) or stroke and baseline cardiovascular risk factors adjusted for all other factors in the model

Hazard ratio (95% CI) for development of				
	MI		Stroke	
Total/ HDL cholesterol (top vs. bottom tertile)	2.80	(2.09, 3.74)**	1.53	(0.99, 2.35)
BMI (1SD increase)	0.95	(0.83, 1.10)	1.24	(1.06, 1.44)**
Pack years of smoking (top vs. bottom tertile)	1.35	(0.96, 1.91)	1.59	(1.07, 2.36)*
Diabetes (yes vs. no)	1.43	(0.91, 2.25)	0.74	(0.38, 1.43)
Leisure activity (no activity vs. strenuous)	1.36	(0.77, 2.41)	1.16	(0.53, 2.56)
ABI (1SD decrease)	1.18	(1.03, 1.33)*	1.18	(1.01, 1.37)*
History of cardiovascular disease (yes vs. no)	2.80	(2.09, 3.74)**	1.60	(1.11, 2.31)*

ABI: ankle brachial index

** p value <0.01

Figure 30 Log -log plot survival curve for major cardiovascular disease (CVD) per tertiles of total cholesterol/ HDL ratio



1: bottom, 2: middle, 3: top tertile
Cut-off points for total/ HDL cholesterol 6.40 and 7.49 mmol/L.

6.10 Inflammatory markers and incident CVD

In this part, the association between inflammatory markers with incident major CVD, MI or stroke was examined.

6.10.1 Descriptive analysis

Table 49 lists the median (interquartile range) of baseline levels of inflammatory markers in people who subsequently developed CVD and in those who remained free of CVD during the follow up. Subjects who developed CVD were further categorised into those who experienced MI (either fatal or non fatal) and those who experienced stroke (fatal or non fatal). Median CRP and IL-6 was consistently higher in people who developed major CVD, MI and stroke compared to the reference group of people with no CVD disease (Table 49). Adhesion molecules, E-selectin ($p = 0.03$) and ICAM-1 ($p = 0.03$) were elevated only in the group who developed stroke compared to the group of people who did not developed CVD. On the other hand, VCAM-1 did not differ significantly across different groups.

6.10.2 Univariable associations with incident CVD

Survival curves for incident major CVD development per tertile of each inflammatory marker are shown in Figure 31 along with the corresponding p value for the log rank test. All inflammatory markers had significant associations with incident CVD and subjects falling in their top tertile of their distributions had increasing risk of developing major CVD. For example, people falling in the top

tertile of CRP or IL-6 compared to those in the bottom tertile had hazard ratio (95% CI) 2.36 (1.75, 3.20) and 2.69 (1.99, 3.65), respectively.

Adhesion molecules showed smaller differences between their tertiles for major CVD survival as illustrated by their survival curves on Figure 31. ICAM-1 and VCAM-1 had hazard ratios (95% CI) 1.56 (1.19, 2.05) and 1.42 (1.08, 1.85) between top and bottom tertile respectively. Finally subjects on the bottom or middle tertile of E-selectin had hazard ratio (95 % CI) for major CVD 1.38 (1.06, 1.79).

Survival curves for MI and stroke separately were essentially unchanged with the exception of E-selectin which showed relatively greater associations with stroke events (hazard ratio (95% CI) 1.67 (1.12, 2.49)) compared to MI (1.28 (0.91, 1.82)).

6.10.3 Multivariable associations with incident CVD

Table 50 presents the hazard ratios (95% CI) between the top and bottom tertile of each inflammatory marker for major CVD and for MI and stroke separately. Hazard ratios were adjusted for age and sex, then conventional risk factors (diabetes, total/HDL cholesterol ratio, BMI, pack years of smoking, physical activity), history of CVD and finally for ABI. CRP and IL-6 had significant hazard ratios for CVD in all analyses; nevertheless, their effect was attenuated after adjustments for conventional risk factors and history of CVD at baseline. Adjustment for subclinical disease after accounting for cardiovascular risk factors did not seem to alter considerably the magnitude of these associations. As shown in Table 50, hazard ratio (95% CI) for people in the top vs. the bottom tertile of CRP at baseline was 1.67 (1.19, 2.34) in

analysis adjusted for CVD risk factors and baseline CVD which was changed to 1.65 (1.18, 2.32) in the multivariable analysis. Likewise, IL-6 had hazard ratio (95% CI) 1.91 (1.37, 2.65) in risk factors and baseline CVD adjusted analysis which was decreased to 1.85 (1.33, 2.58) in the multivariable analysis.

When the effect of CRP and IL-6 was examined in MI and stroke separately some differences were observed. CRP was significantly and independently associated with stroke events with hazard ratio (95 % CI) 1.98 (1.13, 3.46) in the multivariable model. However, its hazard ratio (95% CI) with incident MI was considerably smaller (1.36 (0.89, 2.09)). On the other hand, IL-6 showed comparable effects for MI and stroke.

ICAM-1 had only significant hazard ratios (95% CI) at the age and sex adjusted analysis for major CVD (1.53 (1.16, 2.03)) and for stroke (2.04 (1.29, 3.22)) but not for MI. VCAM-1 did not show any evidence for associations with incident major CVD, MI or stroke. Finally, E-selectin showed significant associations with incident major CVD at the age and sex adjusted analysis (hazard ratio (95% CI) 1.47 (1.12, 1.93)) and with incident stroke even in the multivariable analysis with hazard ratio (95% CI) 1.71 (1.11, 2.64).

When all inflammatory markers were entered simultaneously in the Cox regression model, only IL-6 retained significant associations. Its hazard ratio (95% CI) for major CVD between top and bottom tertile was 1.82 (1.21, 2.76) ($p=0.004$) in

analysis adjusted for age and sex and 1.71 (1.12, 2.61) ($p=0.01$) in multivariable analysis.

The proportional hazard assumptions were tested for all Cox regression models performed. This was done by plotting the log-log curves for each inflammatory markers and looking for non-constant hazard ratios over time. No serious deviations were observed and the log-log plot of IL-6 on Figure 32 is presented as an example. Figure 32 shows that the hazard ratios between tertiles of CRP were approximately constant over time.

6.10.4 Sensitivity analysis

When the analysis was limited to those 1,315 subjects without history of CVD (MI, stroke, angina or IC) at baseline the results did not meaningfully change. CRP and IL-6 had still significant associations with incident major CVD. Their hazard ratios (95% CI) between top and bottom tertile for incident major CVD in analysis adjusted for CVD risk factors and ABI at baseline were 1.58 (1.06, 2.36) and 1.81 (1.22, 2.69) respectively.

Table 49 Median (interquartile range) of inflammatory markers measured at baseline according to group of cardiovascular events occurring during follow up

(a)	N	No CVD (N=1,177)	CVD (N=415)	P value
CRP (mg/L)	992	1.60 (0.84, 3.46)	2.82 (1.13, 5.17)	< 0.001
IL-6 (pg/mL)	1038	2.01 (1.34, 3.28)	2.54 (1.75, 4.17)	< 0.001
ICAM-1 (ng/mL)	1182	214 (186, 257)	220 (192, 266)	0.11
VCAM-1(ng/mL)	1182	370 (320, 430)	376 (335, 446)	0.07
E-selectin (ng/mL)	1179	41 (31, 51)	43 (34, 55)	0.05
(b)	N	No CVD (N=1,177)	Myocardial infarction (N=248)	P value
CRP (mg/L)	992	1.60 (0.84, 3.46)	2.86 (1.10, 5.32)**	0.001
IL-6 (pg/mL)	1038	2.01 (1.34, 3.28)	2.67 (1.72, 4.64)**	<0.001
ICAM-1 (ng/mL)	1182	214 (186, 257)	219 (188, 267)	0.39
VCAM-1(ng/mL)	1182	370 (320, 430)	382 (335, 447)	0.17
E-selectin (ng/mL)	1179	41 (31, 51)	41 (31, 54)	0.35
(c)	N	No CVD (N=1,177)	Stroke (N=168)	p value
CRP (mg/L)	992	1.60 (0.84, 3.46)	3.08 (1.45, 5.42)**	< 0.001
IL-6 (pg/mL)	1038	2.01 (1.34, 3.28)	2.52 (1.81, 4.08)**	0.01
ICAM-1 (ng/mL)	1182	214 (186, 257)	233 (201, 266)*	0.04
VCAM-1(ng/mL)	1182	370 (320, 430)	374 (336, 447)	0.19
E-selectin (ng/mL)	1179	41 (31, 51)	46 (32, 56)*	0.03

CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1.

** p <0.01 *p <0.05.

Figure 31 Survival curves for tertiles for incident major cardiovascular disease (CVD) per tertiles of each inflammatory marker. P values correspond to the log rank test

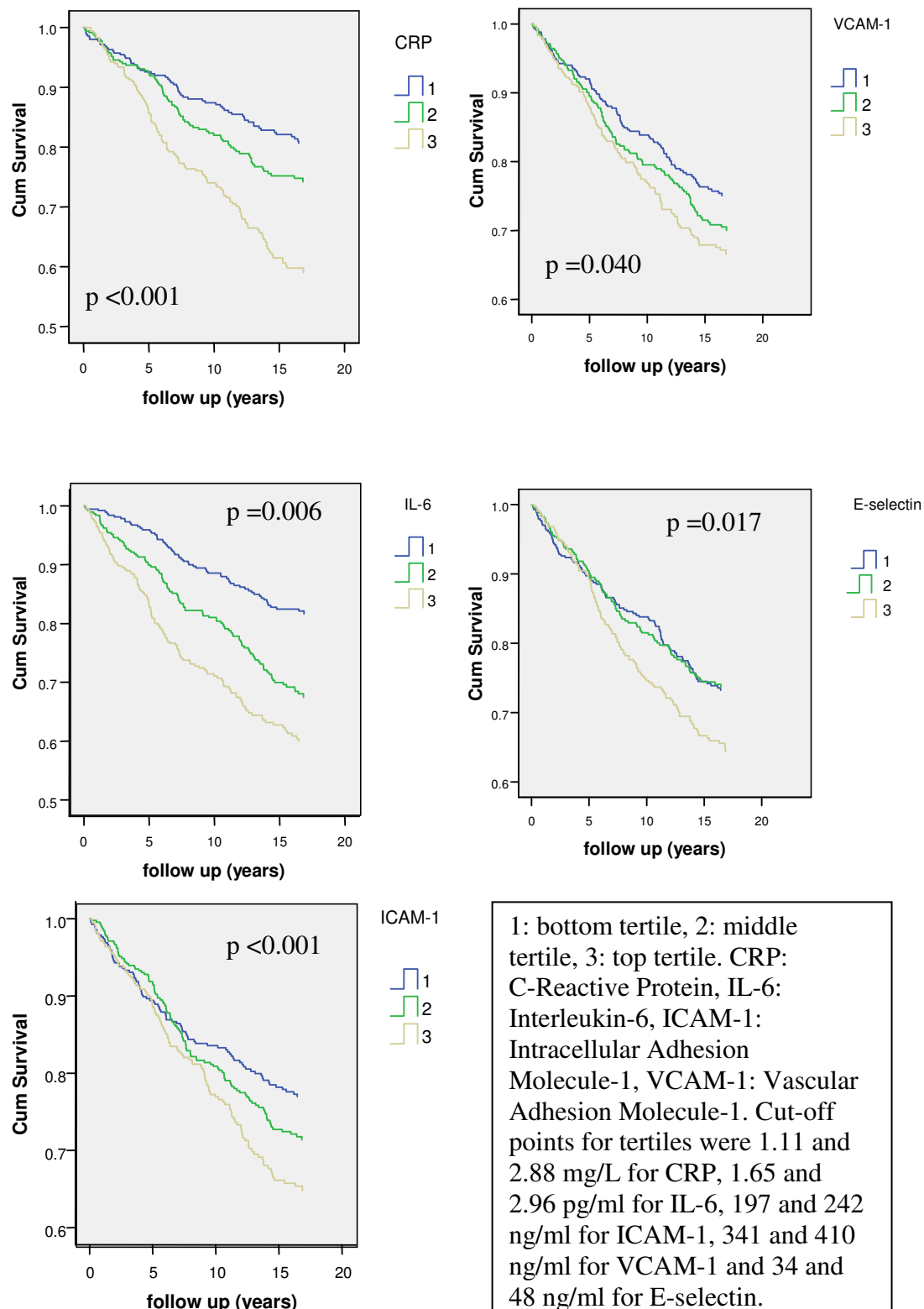
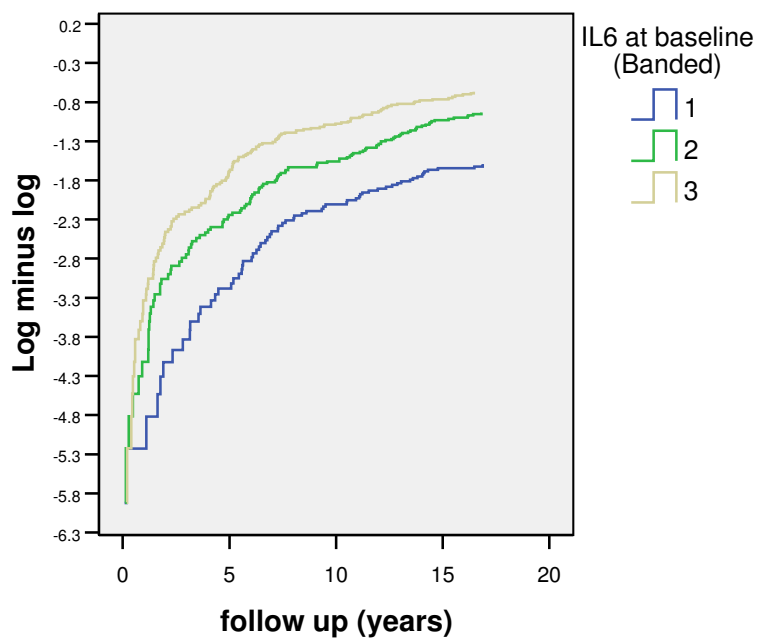


Table 50 Hazard ratios (95 % CI) for (a) cardiovascular disease, (b) myocardial infarction and (c) stroke between top and bottom tertiles of each marker

Adjusted for:	+ age, sex	+ cardiovascular risk factors [†] and history of cardiovascular disease [‡]	+ subclinical disease [§]
<hr/>			
(a)	Cardiovascular disease		
CRP	2.18 (1.59, 2.98)**	1.67 (1.19, 2.34)**	1.65 (1.18, 2.32)**
IL-6	2.31 (2.08, 3.88)**	1.91 (1.37, 2.65)**	1.85 (1.33, 2.58)**
ICAM-1	1.53 (1.16, 2.03)**	1.18 (0.88, 1.59)	1.15 (0.86, 1.55)
VCAM-1	1.27 (0.96, 1.68)	1.21 (0.91, 1.61)	1.22 (0.92, 1.62)
E-selectin	1.47 (1.12, 1.93)**	1.25 (0.95, 1.66)	1.23 (0.93, 1.64)
<hr/>			
(b)	Myocardial infarction		
CRP	1.74 (1.17, 2.58)**	1.37 (0.89, 2.10)	1.36 (0.89, 2.09)
IL-6	2.10 (1.40, 3.16)**	1.78 (1.16, 2.72)**	1.71 (1.11, 2.62)*
ICAM-1	1.43 (1.00, 2.04)	1.15 (0.79, 1.68)	1.12 (0.76, 1.64)
VCAM-1	1.31 (0.91, 1.89)	1.23 (0.85, 1.78)	1.23 (0.85, 1.78)
E-selectin	1.30 (0.91, 1.85)	1.12 (0.77, 1.62)	1.10 (0.76, 1.59)
<hr/>			
(c)	Stroke		
CRP	2.86 (1.69, 4.87)**	2.08 (1.19, 3.62)**	1.98 (1.13, 3.46)*
IL-6	2.35 (1.38, 4.00)**	1.81 (1.04, 3.14)*	1.72 (0.98, 3.00)
ICAM-1	2.04 (1.29, 3.22)**	1.54 (0.96, 2.49)	1.51 (0.93, 2.43)
VCAM-1	1.23 (0.79, 1.93)	1.16 (0.74, 1.82)	1.17 (0.74, 1.83)
E-selectin	2.07 (1.36, 3.15)**	1.74 (1.12, 2.68)**	1.71 (1.11, 2.64)*
<hr/>			
CRP: C-Reactive Protein, IL-6: Interleukin-6, ICAM-1: Intracellular Adhesion Molecule-1, VCAM-1: Vascular Adhesion Molecule-1.			
[†] age, sex, pack years smoking, diabetes, BMI, total/ HDL cholesterol ratio and physical activity.			
[‡] myocardial infarction, stroke or angina.			
[§] ankle brachial index.			

Figure 32 Log -log plot for major cardiovascular disease (CVD) per tertiles of IL-6



1: bottom, 2: middle, 3: top tertile.
Cut-off points for IL-6: 1.65 and 2.96 pg/ml.

6.11 Haemostatic markers and incident CVD

In this part, the association between haemostatic markers with incident major CVD, MI and stroke was examined.

6.11.1 Descriptive analysis

Table 51 lists the median (interquartile range) of baseline levels of inflammatory markers in people who subsequently developed CVD and in those who remained free of CVD during the follow up. Subjects who developed CVD were further categorised into those who experienced MI (either fatal or non fatal) and those who experienced stroke (fatal or non fatal). Median fibrinogen, D-dimer and t-PA were consistently higher in people who developed CVD ($p < 0.001$), MI ($p < 0.001$) and stroke ($p < 0.001$) compared to the reference group of people with no CVD disease (Table 51).

Similarly, median of vWF was significantly higher in people who developed CVD ($p < 0.001$), MI ($p = 0.003$) or stroke ($p = 0.006$). On the contrary, levels of factor VII, FpA and F1+2 did not show any differences in people who did not develop CVD and in those who developed major CVD, MI or stroke.

6.11.2 Univariable associations with incident CVD

Survival curves for incident major CVD development per tertile of each haemostatic marker are shown in Figure 33 along with the corresponding p value for the log rank test. Haemostatic markers fibrinogen, D-dimer, t-PA and vWF had significant (p

<0.001) associations with incident CVD with subjects falling in their top tertile showing increased risk of incident major CVD than those falling in the bottom tertile. Top tertiles of fibrinogen, D-dimer and t-PA were associated with greater than 2 fold increase in the risk of developing major CVD with corresponding hazard ratios (95% CI) 2.23 (1.74, 2.84), 2.26 (1.75, 2.92) and 2.67 (2.03, 3.53), respectively. Levels of vWF had a smaller effect on the risk of major CVD and the hazard ratio (95% CI) between top and bottom tertile was 1.68 (1.31, 2.16).

Factor VII and F1+2 levels did not show any evidence for associations with incident major CVD (Figure 33). Furthermore, subjects on the top tertile of FpA compared to those in the bottom tertile had a small increase in their risk of developing major CVD (hazard ratio (95% CI) 1.29 (1.03, 1.62)).

Survival curves for MI and stroke showed essentially similar results as the combined CVD analysis.

6.11.3 Multivariable associations with incident CVD

Table 52 presents the hazard ratios (95% CI) between the top and bottom tertile of each inflammatory marker for major CVD and for MI and stroke separately. Hazard ratios were adjusted for age and sex, then conventional risk factors (diabetes, total/HDL cholesterol ratio, BMI, pack years of smoking, physical activity), history of CVD, and finally for ABI. Fibrinogen retained significant associations after adjustments for CVD risk factors and baseline CVD and was significantly associated with major CVD and with MI and stroke separately and showed comparable effect

across different disease groups. Further adjustment for subclinical atherosclerosis had small impact on these associations and the hazard ratio (95% CI) for major CVD between top and bottom tertile was 1.85 (1.41, 2.42) in analyses adjusted for CVD risk factors and history of CVD and 1.76 (1.35, 2.31) at the ABI adjusted analysis. Weaker associations were found for D-dimer and incident major CVD, MI or stroke with hazard ratios (95% CI) 1.56 (1.17, 2.09) for major CVD, 1.59 (1.09, 2.33) for MI and 1.53 (0.94, 2.48) for stroke in the multivariable model (top vs. bottom tertile).

Moreover, t-PA had marginally the greatest independent effect for major CVD compared to the other haemostatic markers in the multivariable analysis with hazard ratio (95% CI) 1.86 (1.36, 2.55) (top vs. bottom tertile). Same was true when the analysis was limited to MI events. For stroke events, the effect of t-PA was much limited with hazard ratio (95% CI) 1.47 (0.90, 2.41) (top vs. bottom tertile).

Moreover, vWF presented modest associations with incident major CVD and MI and it did not show any significant associations with future stroke even in the age and sex adjusted analysis. Finally, factor VII, FpA and F1+2 did not show any significant associations either with major CVD or with MI and stroke separately.

When all the haemostatic markers were entered simultaneously into the Cox regression model, t-PA and D-dimer retained significant associations with incident major CVD in analysis adjusted for age and sex only with hazard ratio (95% CI) between top and bottom tertile 2.04 (1.37, 3.03) ($p < 0.001$) and 1.60 (1.03, 2.49) ($p = 0.04$) respectively. However, in the final analysis only t-PA retained significant

associations with hazard ratio (95% CI) between top and bottom tertile 2.01 (1.31, 3.08) ($p=0.001$).

The assumption of proportional hazards was tested for all Cox regression models by plotting the log-log plot for each haemostatic markers and examining the hazard ratios between tertiles over time. For all models no serious deviations were observed. On Figure 34 the log-log plot of fibrinogen is shown as an example. The hazard ratios between tertiles do not differentiate over time.

6.11.4 Sensitivity analysis

When the analysis was limited to those 1,315 subjects without history of CVD (MI, stroke, angina or IC) at baseline the results did not meaningfully change. Fibrinogen, D-dimer, t-PA and vWF had still significant associations with incident major CVD. Their hazard ratios (95% CI) between top and bottom tertile for incident major CVD in analysis adjusted for CVD risk factors and ABI at baseline were 1.69 (1.22, 2.34), 1.63 (1.09, 2.38), 1.61 (1.09, 2.38) and 1.07 (0.78, 1.48), respectively.

Table 51 Median (interquartile range) of haemostatic markers measured at baseline according to group of cardiovascular events occurring during follow up

(a)	N	No CVD (N=1,177)	CVD (N=415)	p value
Fibrinogen (g/L)	1548	2.58 (2.18, 3.01)	2.83 (2.39, 3.34)	< 0.001
D-dimer (ng/mL)	1436	80 (87, 116)	94 (68, 141)	< 0.001
t-PA (ng/mL)	1362	7.0 (5.0, 9.2)	8.4 (6.5, 10.5)	< 0.001
vWF (IU/dl)	1446	105 (78, 137)	115 (87, 148)	< 0.001
Factor VII (IU/dl)	1031	90 (70, 116)	91 (70, 119)	0.41
F1+2 (nmol/L)	1062	1.4 (1.0, 2.0)	1.5 (1.0, 2.0)	0.72
FPA (ng/mL)	1580	1.7 (1.3, 2.1)	1.7 (1.3, 2.1)	0.15
(b)	N	No CVD (N=1,177)	Myocardial infarction (N=248)	p value
Fibrinogen (g/L)	1548	2.58 (2.18, 3.01)	2.86 (2.33, 3.35)	< 0.001
D-dimer (ng/mL)	1436	80 (87, 116)	96 (69, 143)	0.001
t-PA (ng/mL)	1362	7.0 (5.0, 9.2)	8.4 (6.5, 10.7)	< 0.001
vWF (IU/dl)	1446	105 (78, 137)	116 (87, 155)	0.003
Factor VII (IU/dl)	1031	90 (70, 116)	91 (71, 122)	0.32
F1+2 (nmol/L)	1062	1.4 (1.0, 2.0)	1.5 (1.0, 2.0)	0.74
FPA (ng/mL)	1580	1.7 (1.3, 2.1)	1.7 (1.4, 2.1)	0.09
(c)	N	No CVD (N=1,177)	Stroke (N=168)	p value
Fibrinogen (g/L)	1548	2.58 (2.18, 3.01)	2.90 (2.50, 3.43)	< 0.001
D-dimer (ng/mL)	1436	80 (87, 116)	97 (73, 146)	< 0.001
t-PA (ng/mL)	1362	7.0 (5.0, 9.2)	7.9 (6.4, 10.5)	< 0.001
vWF (IU/dl)	1446	105 (78, 137)	117 (87, 157)	0.006
Factor VII (IU/dl)	1031	90 (70, 116)	89 (69, 113)	0.84
F1+2 (nmol/L)	1062	1.4 (1.0, 2.0)	1.4 (1.0, 2.1)	0.85
FPA (ng/mL)	1580	1.7 (1.3, 2.1)	1.7 (1.3, 2.1)	0.97

t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

Figure 33 Survival curves for tertiles for incident major cardiovascular disease (CVD) per tertiles of each haemostatic marker. P values correspond to the log rank test

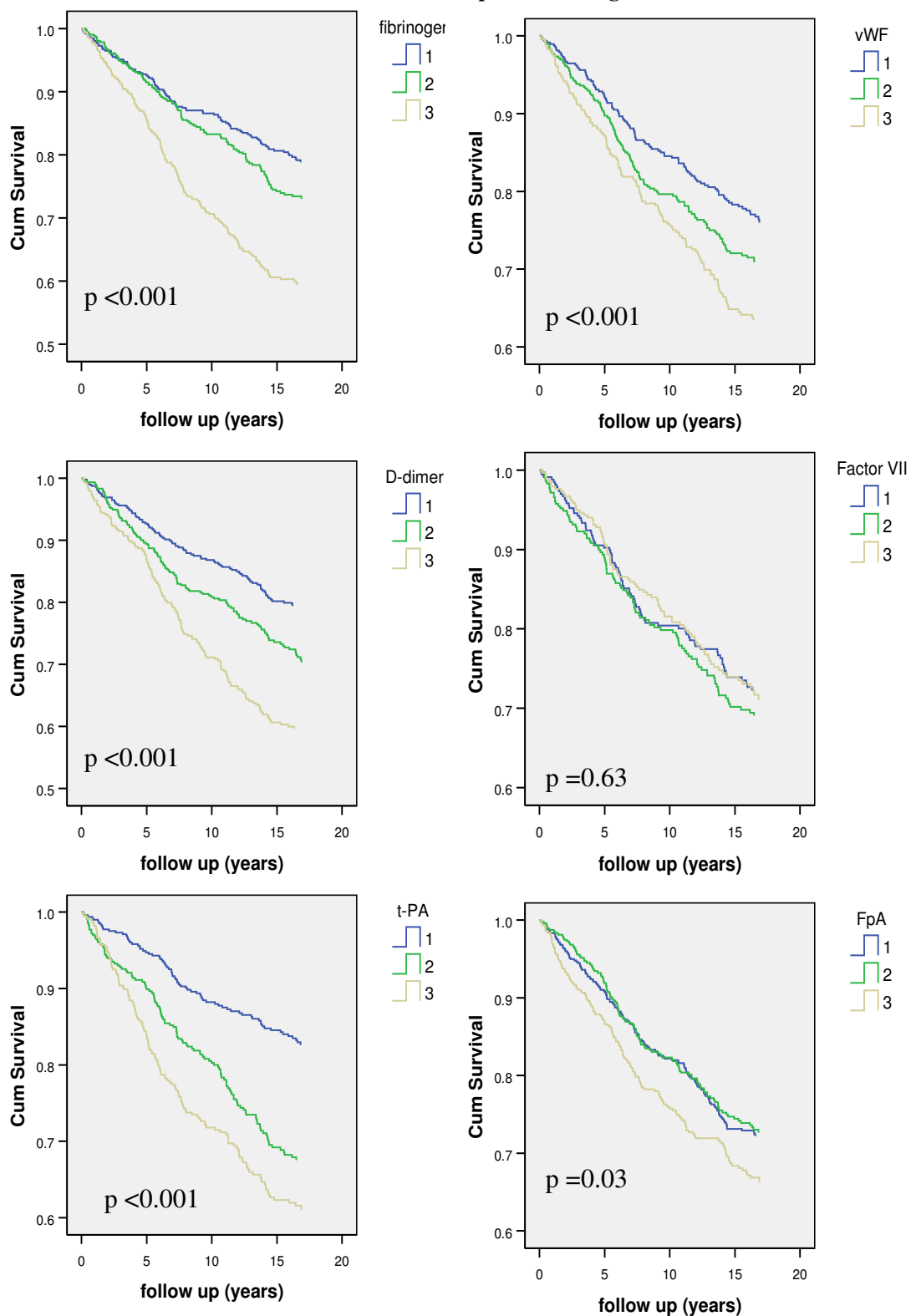
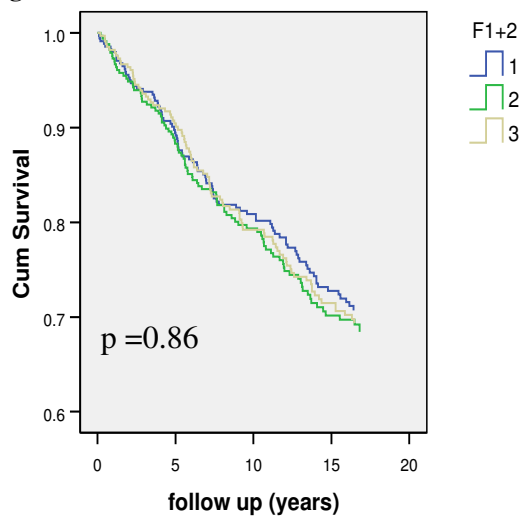


Figure 33 cont.



1: bottom tertile, 2: middle tertile, 3: top tertile.

t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

Cut off point for tertiles: 2.38-2.92 g/L for fibrinogen, 67-107 ng/mL for D-dimer, 6.0-8.8 ng/mL for t-PA, 89-129 IU/dl for vWF, 77-105 IU/dl for Factor VII, 1.48-1.95 ng/mL for F1+2, 1.00-1.70 nmol/L for FpA

Table 52 Hazard ratios (95% CI) for (a) cardiovascular disease, (b) myocardial infarction and (c) stroke between top and bottom tertiles of each marker

Adjusted for:	+ age, sex	+ cardiovascular risk factors [†] and history of cardiovascular disease [‡]	+ subclinical disease [§]
(a)	Cardiovascular disease		
Fibrinogen	2.33 (1.80, 3.01)**	1.85 (1.41, 2.42)**	1.76 (1.35, 2.31)**
D-dimer	1.95 (1.47, 2.57)**	1.65 (1.24, 2.19)**	1.56 (1.17, 2.09)**
t-PA	2.26 (1.68, 3.04)**	1.90 (1.39, 2.60)**	1.86 (1.36, 2.55)**
VWF	1.42 (1.09, 1.85)**	1.34 (1.03, 1.75)*	1.33 (1.02, 1.74)*
F VII	1.05 (0.77, 1.30)	1.01 (0.73, 1.40)	1.01 (0.74, 1.40)
F1+2	1.07 (0.79, 1.46)	1.00 (0.73, 1.36)	1.00 (0.44, 1.37)
FpA	1.16 (0.91, 1.47)	1.12 (0.88, 1.42)	1.11 (0.87, 1.41)
(b)	Myocardial infarction		
Fibrinogen	2.17 (1.57, 3.00)**	1.79 (1.27, 2.52)**	1.70 (1.20, 2.39)**
D-dimer	1.96 (1.36, 2.83)**	1.69 (1.16, 2.47)**	1.59 (1.09, 2.33)*
t-PA	2.16 (1.46, 3.19)**	1.92 (1.27, 2.91)**	1.87 (1.24, 2.83)**
VWF	1.62 (1.15, 2.28)**	1.54 (1.09, 2.18)*	1.53 (1.08, 2.16)*
F VII	1.71 (0.77, 1.77)	1.12 (0.73, 1.71)	1.12 (0.73, 1.71)
F1+2	1.00 (0.67, 1.49)	0.92 (0.62, 1.38)	0.92 (0.61, 1.37)
FpA	1.38 (1.01, 1.89)*	1.36 (0.99, 1.87)	1.36 (0.99, 1.87)
(c)	Stroke		
Fibrinogen	2.62 (1.70, 2.03)**	2.08 (1.33, 3.24)**	1.95 (1.25, 3.06)**
D-dimer	1.98 (1.24, 3.15)**	1.63 (1.01, 2.62)*	1.53 (0.94, 2.48)
t-PA	2.00 (1.26, 3.18)**	1.50 (0.91, 2.45)	1.47 (0.90, 2.41)
VWF	1.10 (0.73, 1.65)	1.04 (0.69, 1.56)	1.02 (0.68, 1.54)
F VII	0.94 (0.57, 1.55)	0.85 (0.51, 1.42)	0.86 (0.51, 1.43)
F1+2	1.03 (0.63, 1.67)	0.95 (0.58, 1.55)	0.98 (0.60, 1.60)
FpA	0.83 (0.59, 1.24)	0.85 (0.58, 1.24)	0.83 (0.57, 1.21)

t-PA: tissue plasminogen, vWF: vonWillebrand factor, FpA: fibrinopeptide A, F1+2: prothrombin fragments 1+2.

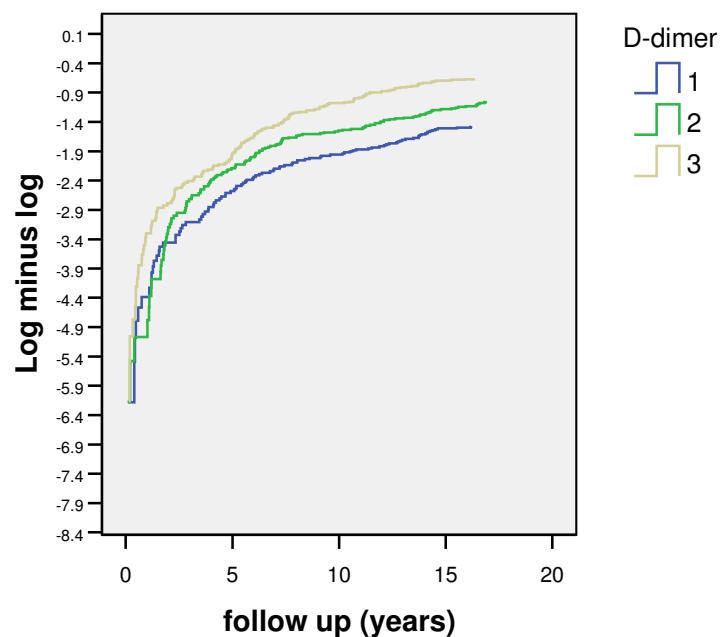
* p <0.01, ** p <0.05.

[†] age, sex, pack years smoking, diabetes, BMI, total/ HDL cholesterol ratio and physical activity.

[‡] myocardial infarction, stroke or angina.

[§] ankle brachial index.

Figure 34 Log-log survival plot for major cardiovascular disease (CVD) per tertiles of D-dimer



1:top, 2:middle, 3:bottom
Cut-off points for D-dimer: 67 and 107 ng/ ml

6.12 Additive effect of inflammatory markers and haemostatic markers on incident CVD

In this part, the additive effect between inflammatory and haemostatic markers on their associations with incident major CVD was investigated.

6.12.1 Composite score: IL-6, fibrinogen and t-PA

The previous analysis showed the inflammatory markers CRP and IL-6 and haemostatic factor fibrinogen, D-dimer and t-PA were independent predictors of major CVD after 17 years of follow up. In particular, IL-6, fibrinogen and t-PA had the highest hazard ratio associated with 1.8 fold or greater increase in risk of major CVD development (upper vs. lower tertile). Next, all variables that have shown a significant effect with incident CVD in the univariable analysis were entered in the Cox regression model with backward selection. In this analysis, cardiovascular risk factors, history of CVD at baseline and ABI were forced to enter in the model. Interestingly, IL-6, fibrinogen and t-PA were the only inflammatory/ haemostatic markers that stayed in the model.

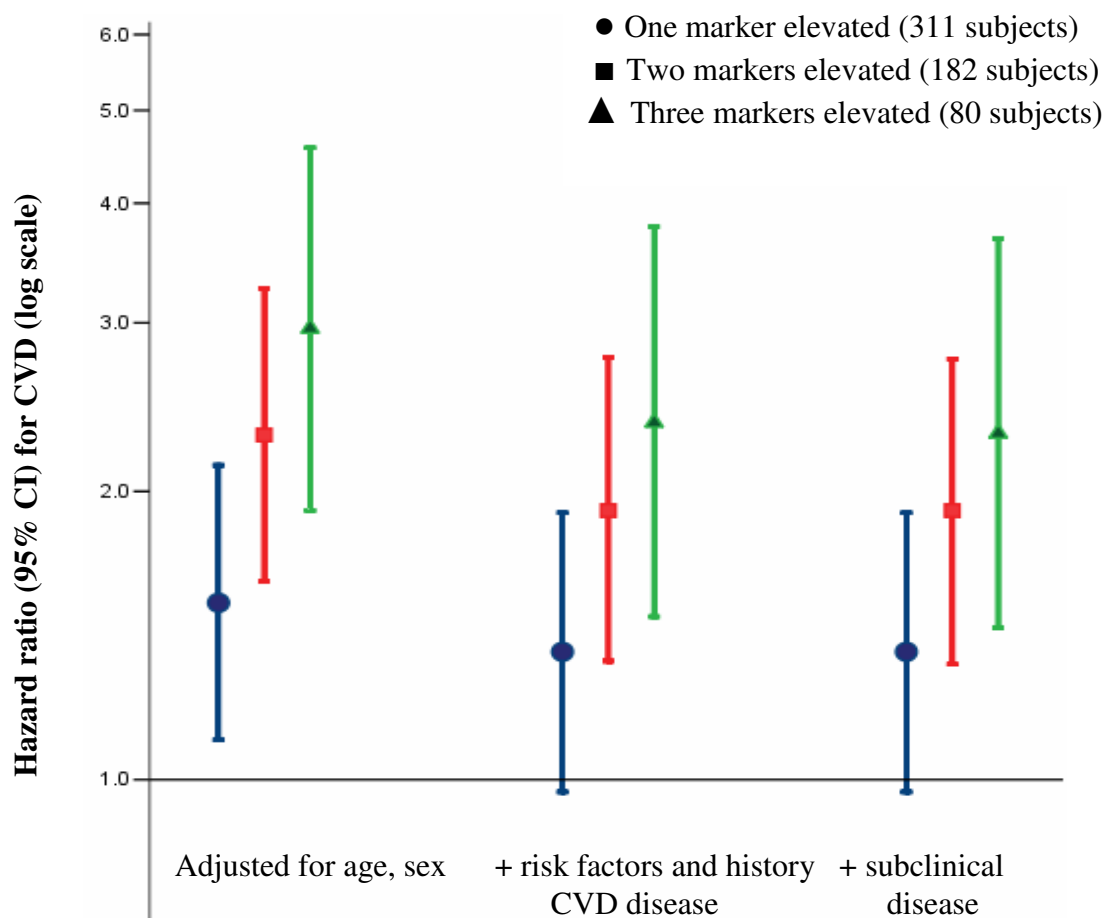
In order to examine the impact of concomitant variables on the long term risk of major CVD a composite score for elevated concentrations of IL-6, fibrinogen and t-PA was calculated. Five percent of the population had all 3 markers in the top tertile, 11.4% had 2 markers and 19.5% one marker. The risk of major CVD rose with increasing number of these markers within the top tertile of their distribution (Figure 35). People having three markers elevated compared to those having none

had hazard ratio (95% CI) 2.30 (1.44, 3.67) ($p=0.001$) of developing major CVD. This was independent of conventional risk factors, history of disease and baseline ABI. People having 2 and 1 markers elevated only compared to those who had none elevated had hazard ratio (95% CI) 1.85 (1.287, 2.69) ($p < 0.001$) and 1.37 (0.97, 1.92) ($p = 0.07$).

6.12.2 Combined variables: IL-6 and D-dimer

Recall that IL-6 and D-dimer had shown an additive effect in relation to progression of peripheral atherosclerosis in previous analysis. Here this additive effect was examined in relation to major CVD. Individuals were divided into 4 mutually exclusive groups according to their baseline levels of D-dimer and IL-6: levels of IL-6 and D-dimer in the top tertile (171 subjects), levels of IL-6 in the top tertile and levels of D-dimer in the middle or bottom tertile (180 subjects), levels of D-Dimer in the top tertile and levels of IL-6 in the middle or bottom tertile (170 subjects) and finally levels of both D-dimer and IL-6 in the middle or bottom tertile (506 subjects). The hazard ratio (95% CI) for people having both IL-6 and D-dimer at the top tertile compare to the group of subjects with neither of them at the top tertile was 1.70 (1.20, 2.39) ($p = 0.003$) in analyses adjusted for CVD risk factors, history of CVD and subclinical atherosclerosis. Similarly, people having IL-6 elevated only or D-dimer elevated only had hazard ratio (95% CI) compared to subjects with neither of them elevated 1.51 (1.07, 2.13) ($p=0.01$) and 1.31 (0.90, 1.90) ($p=0.11$) respectively. The p value for trend was highly significant ($p < 0.001$) and therefore the additive effect between IL-6 and D-dimer observed in analysis for ABI change in the previous chapter was also confirmed here in relation to incident major CVD.

Figure 35 Hazard ratio (95% CI) for CVD associated with increasing numbers of elevated (top tertile) markers (IL-6, fibrinogen or t-PA)



The hazard ratio of CVD was computed using subjects with baseline levels of IL-6, fibrinogen and t-PA at the bottom tertile as the reference group. Risk factors included BMI, diabetes, pack years of smoking, total/ HDL cholesterol ratio and physical activity. History of CVD included myocardial infarction, stroke, angina or IC at baseline. Subclinical atherosclerosis was assessed using the ankle brachial index.

6.13 Inflammatory and haemostatic markers and incident CVD according to cardiovascular risk factors status at baseline

In the previous analysis on peripheral atherosclerotic progression and on incident PAD, the associations between inflammatory and haemostatic markers CRP, IL-6, D-dimer and fibrinogen has been examined according to major cardiovascular risk factors status at baseline. Here the analysis is repeated on incident major CVD for comparison.

6.13.1 Cholesterol levels

The baseline population was stratified according to total/ HDL cholesterol levels and CRP into 6 groups: people with total/ HDL cholesterol ratio below the median and with CRP at bottom, middle and top tertile and people with total/ HDL cholesterol ratio above the median with CRP at bottom, middle or top tertile. Similarly, 6 other groups were created according to IL-6 and total/ HDL cholesterol ratio, fibrinogen and total/ HDL cholesterol ratio or D-dimer and total/ HDL cholesterol ratio.

Figure 36 illustrates the hazard ratios (adjusted for cardiovascular risk factors, history of CVD and ABI) for each group compared to the group of subjects with total/ HDL cholesterol below the median and either, CRP, IL-6, fibrinogen or D-dimer at the bottom tertile (reference group). As the figure shows, subjects with increased levels (top tertile) of any of these 4 variables had increased risk of developing major CVD especially if their cholesterol/ HDL ratio was above the

median. If their cholesterol levels were below the median, increased levels of CRP, IL-6 and D-dimer were still significantly associated with incident major CVD but their effect was reduced. Levels of fibrinogen showed similar effects on incident CVD whether the total/ HDL cholesterol ratio was below or above the median with hazard ratio 2.00 (1.33, 2.99) and 1.87 (1.29, 2.76) (top tertile vs. reference group) respectively.

6.13.2 Smoking

The baseline population was stratified according to smoking status (never and current/ ex smokers) and CRP into 6 groups: never smokers and CRP at bottom, middle and top tertile and current or ex smokers and CRP at bottom, middle or top tertile. Similarly, 6 other groups were created according to IL-6 and smoking status, fibrinogen and smoking status or D-dimer and smoking status.

Figure 37 illustrates the hazard ratios (adjusted for cardiovascular risk factors, history of CVD and ABI) for each group compared to the group of never smokers with CRP, IL-6, fibrinogen or D-dimer at the bottom tertile (reference group). As the figure shows, there was little difference on the effect of these markers according to smoking status. The hazard ratios for the top tertile of CRP, IL-6 or fibrinogen were approximately the same whether subjects were never or current/ ex smokers. Only D-dimer showed small differences, its hazard ratio (95% CI) in never smokers was only 1.32 (0.76, 2.28) (top tertile vs. reference group) but among current or ex smokers it was considerably higher (2.17 (1.36, 3.46)).

Figure 36 Hazard ratios (95% CI) for major cardiovascular disease (CVD) according to tertiles of C-reactive protein (CRP), interleukin-6 (IL-6), fibrinogen or D-dimer and total/ HDL cholesterol

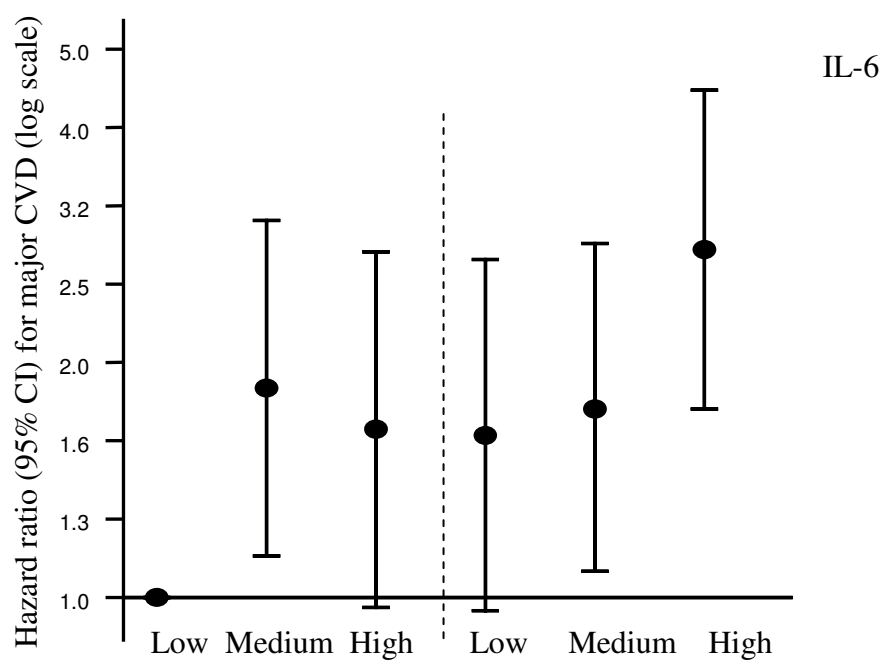
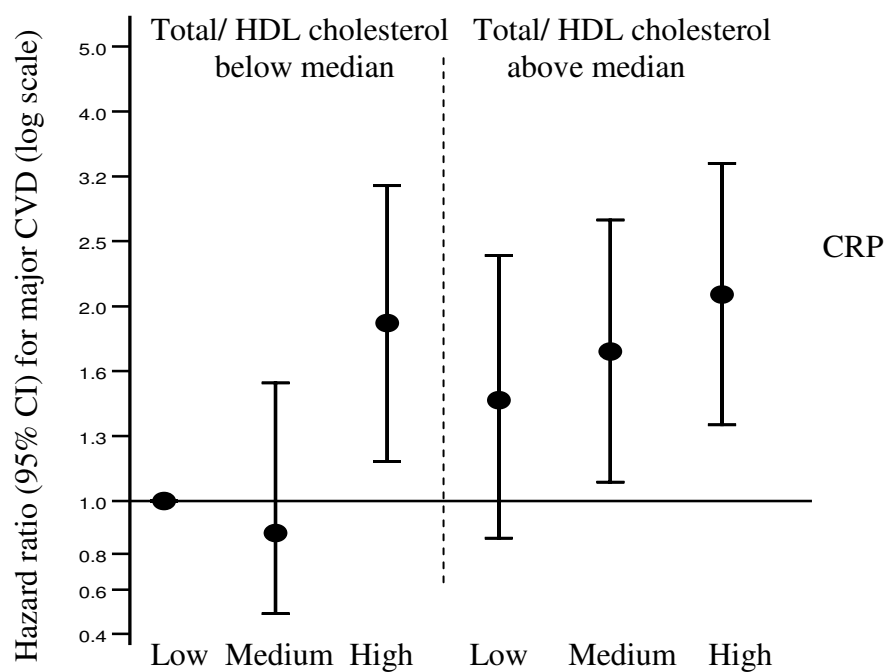
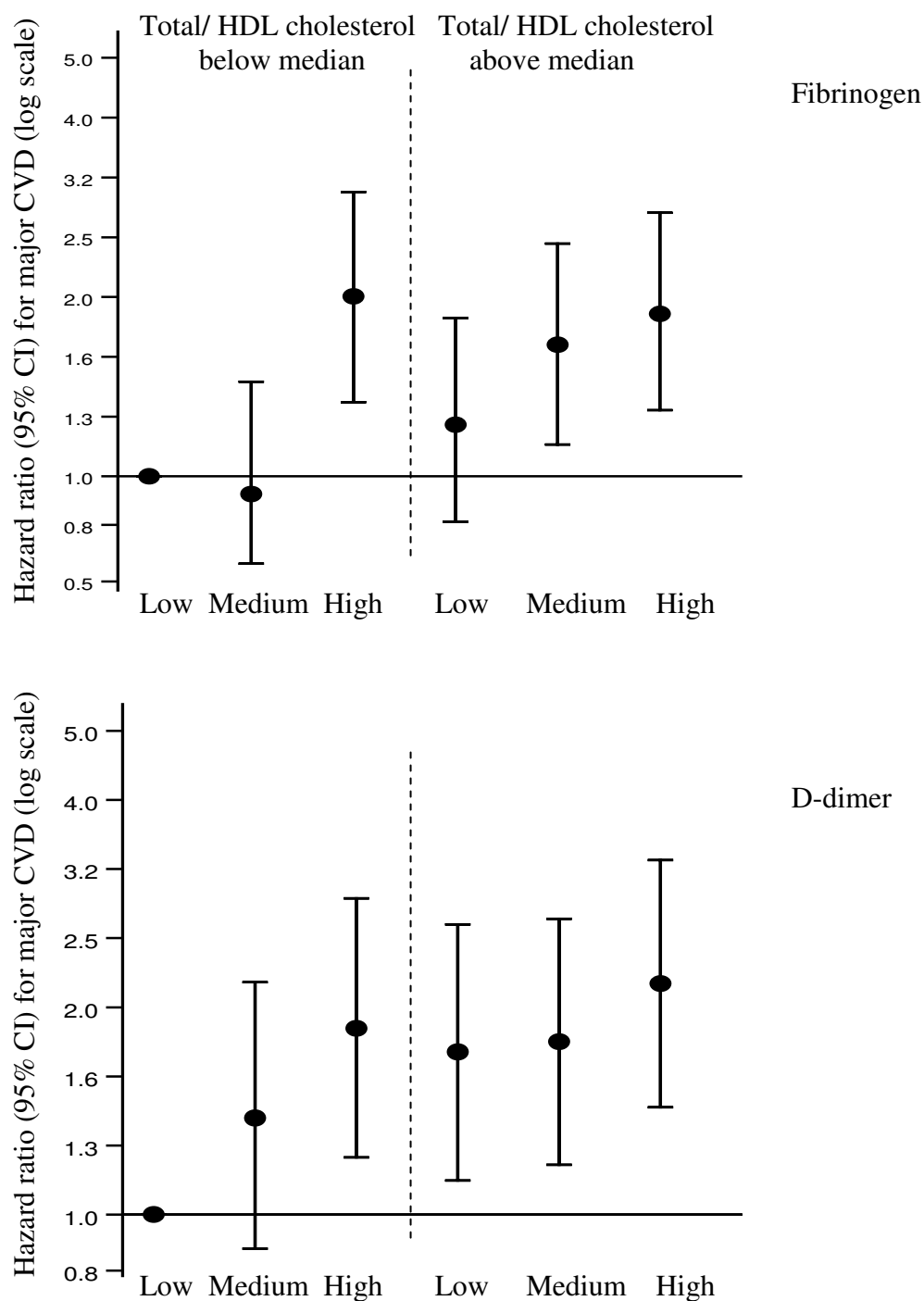


Figure 36 cont.



The hazard ratios are adjusted for age, sex, BMI, diabetes, pack years smoking, physical activity, cardiovascular disease and ankle brachial index. Reference group was the group of people with CRP, IL-6, fibrinogen or D-dimer at the bottom tertile and total/ HDL cholesterol below the median. Median for total/ HDL cholesterol: 4.96. Cut-off point for tertiles of CRP: 1.13, 3.07 mg/L, IL-6: 1.65, 2.69 ng/ml, fibrinogen 2.38, 2.92 g/L and D-dimer 67, 107 ng/ ml.

Figure 37 Hazard ratios (95% CI) for major cardiovascular disease (CVD) according to tertiles of C-reactive protein (CRP), interleukin-6 (IL-6), fibrinogen or D-dimer and total/ HDL cholesterol

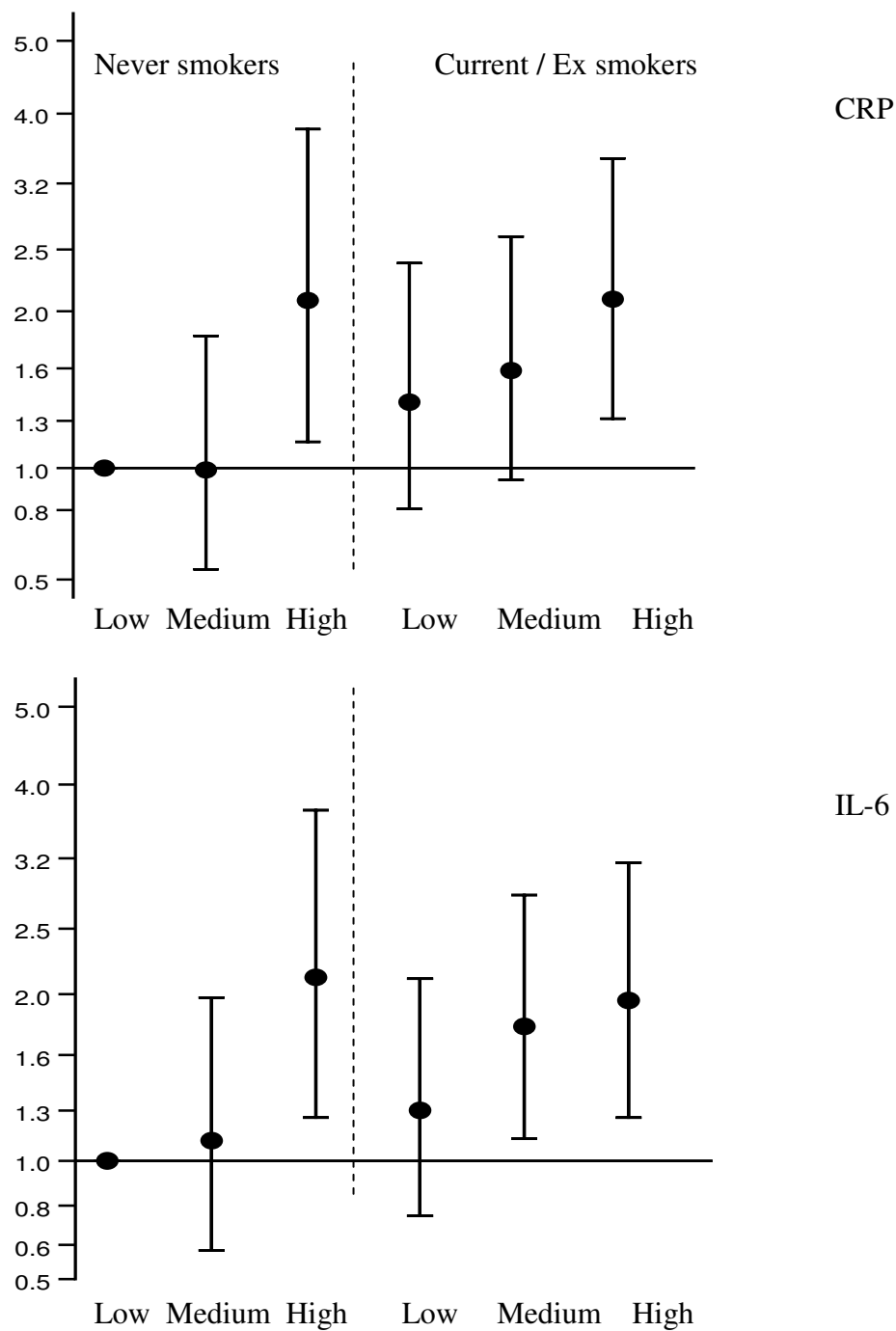
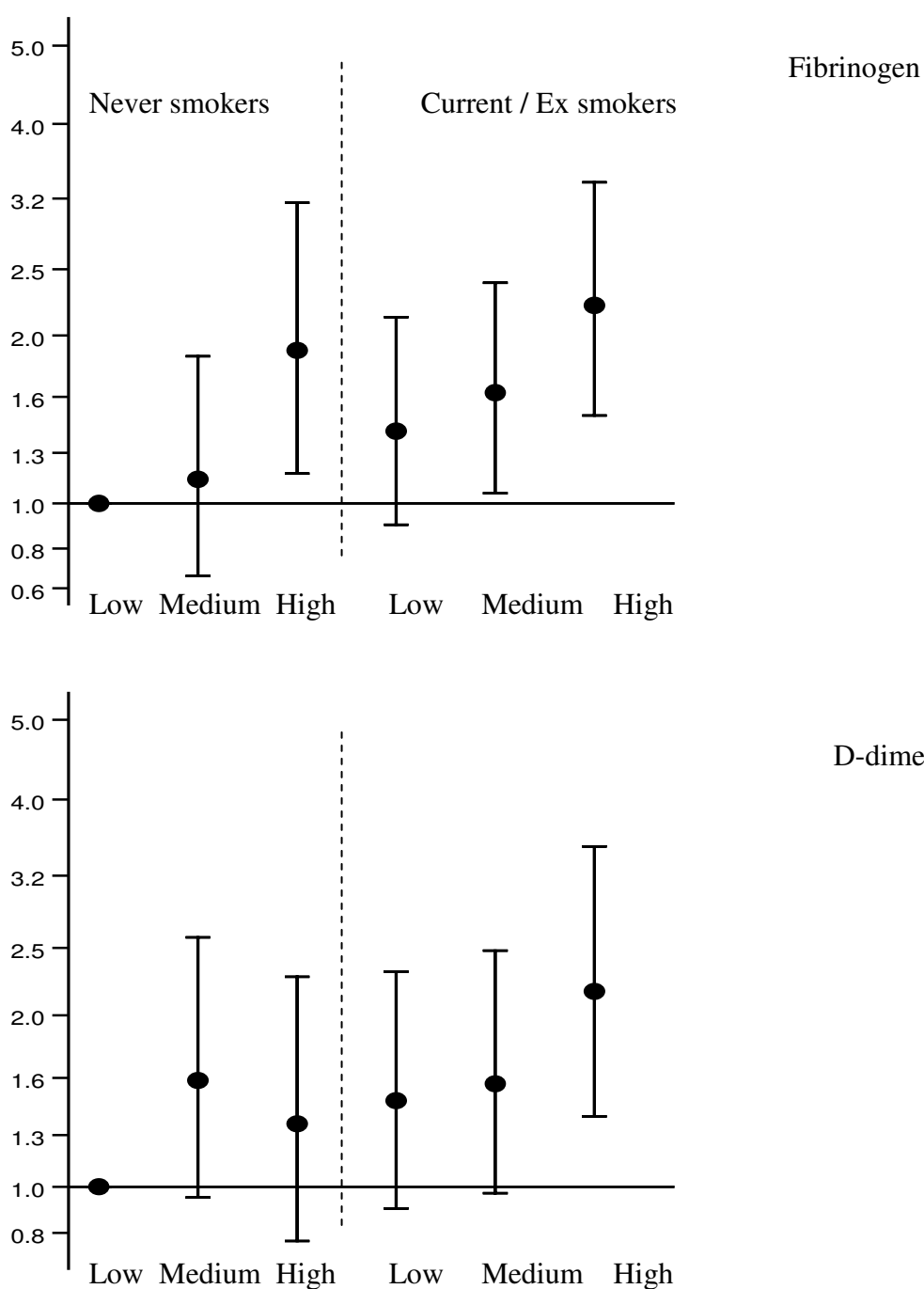


Figure 37 cont.



The hazard ratios are adjusted for age, sex, BMI, diabetes, pack years smoking, physical activity, cardiovascular disease and ankle brachial index. Reference group was the group of people with CRP, IL-6, fibrinogen or D-dimer at the bottom tertile and never smokers. Never smokers: 561 and current/ ex smokers 986 subjects. Cut-off point for tertiles of CRP: 1.13, 3.07 mg/L, IL-6: 1.65, 2.69 ng/ml, fibrinogen 2.38, 2.92 g/L and D-dimer 67, 107 ng/ ml.

6.14 Summary of results

Approximately 14% of the baseline population developed PAD defined as IC, CLI or surgical intervention after 17 years of follow up. Smoking, total/ HDL cholesterol, hypertension and pre-existing CVD ($p < 0.01$) were strong and independent predictors of incident PAD. Inflammatory markers, CRP and IL-6 showed modest associations with PAD. On the other hand, ICAM-1, fibrinogen and D-dimer ($p < 0.01$) had stronger associations which were independent of cardiovascular risk factors and baseline CVD. In particular, fibrinogen had the greatest independent effect compared to other markers, which was not changed in the absence of increased cholesterol or smoking. Other inflammatory and haemostatic markers examined here did not show any significant associations with incident PAD.

Moreover, 26% of the baseline population developed at least one event of major CVD defined as fatal/ non-fatal MI, fatal/ non fatal stroke, CABG or angioplasty. CRP, IL-6, ICAM-1, fibrinogen, D-dimer and t-PA ($p < 0.01$) had significant associations with major CVD which were independent of cardiovascular risk factors and history of CVD. Further adjustments for subclinical atherosclerosis measured by the ABI little affected these associations. Also their effect was little changed in the absence of high cholesterol or in never smokers. Other markers did not show significant associations with major CVD. An incremental risk between people with one, two or all of fibrinogen, IL-6 or t-PA at the top tertile was found. Finally, when the events of MI or stroke were examined separately, CRP and E-selectin had relatively stronger associations with stroke.

Chapter seven

7 Discussion

7.1 Introduction

The aforementioned analysis described results from the Edinburgh Artery Study on the associations between inflammatory and haemostatic markers with the presence and progression of PAD. The principal finding of this study was that several inflammatory and haemostatic markers showed associations with progression of peripheral atherosclerosis and with incident PAD. CRP, IL-6, fibrinogen and to a lesser extent D-dimer and ICAM-1 showed the strongest and most consistent associations. In this section, the principal results of the analysis described in this thesis are discussed. Prior to this discussion, the strengths and limitations of the methods employed are evaluated.

7.2 Strengths and limitations

7.2.1 Strengths

Analyses presented in this thesis are based on the Edinburgh Artery Study. This is a relatively large cohort study of men and women almost exclusively of white origin who lived in Edinburgh. Sampling was population based reducing the bias from selected, hospital based recruitment. Other advantages of the Edinburgh Artery Study included the equal representation of both men and women across a range of adult groups and the long duration of follow up. A variety of risk factors were measured at baseline which enabled the adjustment for several confounders. All risk factor

measurements were performed using standard protocols and strict quality control measures.

Another major strength of this cohort is its prospective design. The subjects were followed up for 17 years for cardiovascular events and all cause mortality. Most importantly, 2 clinical examinations after the baseline examination took place after 5 and after 12 years. The latter enabled the measurement of the ABI over three time points for the first time in the general population. The events ascertainment was ensured by a standardized quality assurance protocol. Also, a key strength of the Edinburgh Artery Study was the careful follow up of the subjects via multiple methods. The Information Statistics Division of the Scottish Executive (Scotland's national organization for health information) was used which collects amongst the best health service data in the world. This service is able to link data to allow patient based analysis and follow up and therefore provides high quality and reliable data of national coverage (Scottish Health Statistics 2006). Subjects of the Edinburgh Artery Study would be lost to follow up only if they had left the country (UK) and this number is expected to be very small. Also, information on cardiovascular events was collected from general practitioners and retrieval of hospital records as well as from the subjects themselves via annual questionnaires.

Moreover, the Edinburgh Artery Study ranks among the few epidemiological studies that focused on PAD. Contrary to many other cohorts which evaluated PAD only by the ABI or by the WHO IC questionnaire, here a variety of diagnostic methods were used including the ABI, the WHO IC questionnaire and most importantly the

assessment of more severe forms of PAD such as rest pain, gangrene, ulceration and surgical intervention. A further strength was the evaluation of incident PAD events in the general population since many studies on PAD have either a cross sectional design or focus just on patients with IC.

7.2.2 Limitations

Measurement of inflammatory and haemostatic markers

The inflammatory and haemostatic markers were measured only once, and intra-individual variation therefore could not be taken into account. Nevertheless, this would tend to result in an underestimation of the true effects, yielding underestimates of the actual risk associated with these measures.

Moreover, not all subjects had measurements for all of the haemostatic and inflammatory markers studied here. Data on some markers is missing due to attrition or decreasing availability of the plasma sample. This was mostly due to the volume of the original sample and would not be expected to be associated with the demographic and cardiovascular risk profile of the patients. Thus, the data was considered as missing ‘at random’. This can be further explored by examining the distribution of cardiovascular risk factors of the cohort in subjects with and without missing markers, for example CRP. As expected, the numbers fluctuated slightly between the two groups but no major differences were observed (Table 12). Also, ‘missingness’ was not associated with incidence of CVD. For example, 26% of those who experienced at least one cardiovascular event had missing CRP values. On the

other hand, 28% of those who did not experience a cardiovascular event had missing CRP values.

Measurement of ABI

Another limitation is that data to adjust for measurement error in ABI were not available; again, this probably led to an underestimation of the strength of the reported associations. Also, for the ABI change analysis, participants in the 5 and 12 year follow up examinations were probably healthier at baseline than those who died during the follow up period. This might explain the higher baseline ABI (1.07) of those 747 subjects who attended all 3 clinical examinations. However, the trend in ABI decline during the 12 years between the individual examination attenders and the group who attended all 3 clinical examinations was similar and suggests that the findings and conclusions are likely to be valid.

Incident disease

Most studies on PAD have defined the disease according to the presence of symptoms of IC only. In the present study the more severe events of CLI and surgical interventions were included to increase the generalizability of the findings. However, asymptomatic disease as defined by the ABI was not included because this measurement was not available after 17 years of follow up. Therefore the incidence of PAD is probably underestimated.

General limitations

The generalization of these results to other ethnic populations and age groups is unknown. Furthermore, the analyses were not adjusted for aspirin or statin use at baseline; however, at the time of baseline examination (1987/1988), very few of the Edinburgh population took such drugs for the prevention of CVD.

Despite the prospective design of this study, an observational study cannot establish causal relations, and residual confounding cannot be ruled out. Therefore, the precise pathway through which inflammation and haemostasis influence atherosclerotic progression and the directionality of the reported associations remain unknown.

Similarly, non-significant associations may have resulted if inflammatory/haemostatic markers exerted their effect on the disease outcome via cardiovascular risk factors like diabetes or BMI. If this was true, these variables should not be used as covariates in the model because they were not confounders but intermediary factors.

7.3 Progression of peripheral atherosclerosis and incident PAD

In this population cohort study, the progression of peripheral atherosclerosis was assessed by the change in ABI between 3 time points over a 12 years follow up period. Although the mean changes in the whole population were subtle, the ABI decreased significantly over the years. This demonstrates the progression of peripheral atherosclerosis over time in the study population and their increasing risk of developing CVD since the ABI is a reliable and accurate marker of generalized atherosclerosis in populations (Bird et al 1999; Fischer et al 1996).

The measurement of ABI as a quantitative measure of disease over time has not been thoroughly investigated before. In the San Diego study, the ABI was measured at baseline and after 5 years of follow up and a progression was defined by the highest tertile of decline which was a -0.30 ABI (Aboyans et al 2006). Approximately 11% of the baseline population with valid ABI on both legs had progression of disease under this definition. The mean (SD) change in the whole population was -0.06 (0.15) after 5 years which is a little higher than the -0.04 (0.18) (also at five years) observed in our cohort. A decrease of -0.014 per year has been reported in claudicants (Aquino et al 2001).

Also, among those with an $\text{ABI} > 0.9$ at baseline, 13% and 18% had an $\text{ABI} \leq 0.9$ at 5 and 12 years, respectively. This incidence of asymptomatic PAD is difficult to compare with other studies because of lack of data and differences in the use of diagnostic methods to assess asymptomatic disease. In the Limburg PAOD study, the incidence rate of an $\text{ABI} \leq 0.9$ among people with $\text{ABI} > 0.9$ at baseline was 9.9/1,000 person years (Hooi et al 2001). This is slightly smaller than the one reported here but in the present analysis, contrary to the Limburg analysis, people who developed IC during the follow up were excluded. Moreover, the Edinburgh Artery Study population is relatively older than that of the Limburg cohort so that an increased incidence of disease might be expected.

Over a longer time period of 17 years, approximately 14% of the study population (with no symptomatic PAD at baseline) developed clinical PAD (82 per 10,000 population per year). In general, our results are higher than annual rates reported

from the Framingham Heart Study (61 per 10,000 men and 54 per 10,000 women per year aged 64-74) (Kannel et al 1970) or the Quebec Cardiovascular Study (41 per 10,000 population per year) (Dagenais et al 1991). However, these studies only included IC in the definition of PAD and not rest pain, ulceration, gangrene and surgical intervention which were recorded in the Edinburgh Artery Study. Therefore, it would be expected that the incidence of clinical PAD in this cohort would be higher than in the aforementioned studies.

7.4 Cardiovascular risk factors and PAD

Risk factors for progression of subclinical PAD were older age, male sex, smoking, hypertension, total/ HDL cholesterol ratio and history of CVD. Only one other study, the Cardiovascular Health Study, has evaluated risk factors for decline in ABI in the general population (Kennedy et al 2005). Progression of asymptomatic PAD was defined as a decline of a participant's ABI of more than 0.15 and to 0.9 or less and the predictors of ABI decline were age, current cigarette use, hypertension, diabetes, higher LDL cholesterol level and lipid-lowering drug use. These results are in accordance with those reported here with the exception of diabetes which failed to show significant associations with ABI decline in the present study.

Risk factors for subclinical PAD were similar to those for clinical PAD. Age, male sex, smoking, total/HDL cholesterol, hypertension and pre-existing CVD were strong independent predictors of PAD development, as previously described (Murabito et al 1997, Fowkes et al 1992). Despite the higher prevalence of diabetes in the group of people who developed PAD compared to those who did not develop PAD, the

difference did not reach statistical significance. The absence of significant associations between diabetes with the progression of peripheral atherosclerosis and with incident PAD is surprising because diabetes is considered an important risk factor for PAD. The non-significance of the reported associations might thus be due to low statistical power and does not necessarily mean that presence of diabetes is not important for the progression and incidence of PAD.

Physical activity has not been extensively investigated in relation to PAD development. Here there was not any clear association between decreased physical activity and PAD development. BMI also showed no evidence for associations with incident PAD. Interestingly it showed borderline significant associations with ABI decline after 12 years of follow up but in the opposite direction. Given that high BMI and obesity are risk factors for CVD this result is probably a statistical artifact especially since it was only significant in the multivariable and not the univariable analysis.

7.5 C-reactive protein

7.5.1 C-reactive protein and subclinical disease

The main aim of this analysis was to examine the association of inflammatory markers with the progression of atherosclerosis. CRP is probably the most extensively studied inflammatory marker in relation to CVD (Koenig 2005). The present analysis showed that CRP was also associated with progressive atherosclerosis measured by the ABI decline after 5 and after 12 years of follow up.

Most importantly, these associations changed little after adjustment for conventional risk factors and the presence of clinical CVD at baseline. This result shows that CRP is associated with atherosclerotic development and that this association is at least not entirely due to interrelations between CRP, cardiovascular risk factors and CVD. Associations between CRP and the extent of atherosclerotic disease have shown conflicting results in previous studies. In general, there is little information on CRP and ABI and most research has focused on other markers of subclinical disease such as the IMT. Only two other prospective studies exist in the literature. In the Rotterdam study, subclinical atherosclerosis was assessed at various sites of the arterial tree at 2 points in time, with a mean duration between measurements of 6.5 years (van der Meer et al 2002b). Data from this analysis showed that CRP levels increased with the total number of sites showing progression of atherosclerosis. The odd ratio for generalized progression of atherosclerosis as indicated by the composite progression score was 4.5 (95% CI, 2.3 to 8.5) which was as high as those associated with the traditional cardiovascular risk factors high cholesterol, hypertension and smoking.

Moreover, in relation to progressive carotid atherosclerosis measured by IMT change, Hashimoto et al, demonstrated that CRP concentration was an independent predictor of the development of early atherosclerosis and that it was associated with the rate of plaque development (Hashimoto et al 2004). However, the latter study was conducted among hypertensive patients being managed by drug therapy or lifestyle modification and had a short term follow up of 35 months. Interestingly, CRP was an equivalent or better independent predictor of the progression of carotid

atherosclerosis than the pulse pressure or systolic blood pressure (Hashimoto et al 2004).

Results from two cross-sectional population studies, the Dallas Heart Study and the Framingham Heart Study, agreed that measured CRP levels were associated with a modest increase in the prevalence of subclinical coronary atherosclerosis as measured by electron beam computerized tomography for coronary calcium (Khera et al 2006; Wang et al 2002). However, the reported associations were not independent of traditional cardiovascular risk factors. In support, CRP was not associated with ABI in the multivariable analysis among participants without a history of CVD in another cohort (McDermott et al 2003). Also, modestly increased CRP levels were observed in male subjects with aortic plaque in the Dallas Heart Study; although this relationship was no longer evident after adjustment for other risk factors (Khera et al 2006). Similarly, data from the Health ABC study showed that CRP was not significantly associated with overall subclinical CVD defined as positive findings on the WHO questionnaire for angina or claudication, ABI<0.9, or electrocardiographic abnormalities (Cesari et al 2003). In a Danish cohort, CRP was associated with IMT levels but not with the number of carotid plaques (De Maat et al 2003).

CRP was also strongly associated with ABI decline in subjects with elevated risk factors at baseline (cholesterol, smoking) in the present study. On the other hand, in the absence of these factors, the associations between CRP and ABI decline were modest. Similar findings were observed when CRP was associated with incident

CVD; the effect of CRP on incident CVD among subjects with low cholesterol levels was attenuated. Several studies have observed that among 'low' risk individuals (according to a combination of conventional cardiovascular risk factors) high CRP levels do not indicate high CVD event rates (Koenig et al 2004; Ridker et al 2002; van der Meer et al 2003). Therefore, results of the present and past studies reinforce evidence for a potential limited clinical value of CRP in individuals at high risk on the basis of traditional risk factors. It should be stressed however, that given that CRP was associated (even weakly) with progression of atherosclerosis and incident CVD among non-smoker subjects or among subjects with low cholesterol, it is more likely to have a causal role in disease development and progression.

7.5.2 C-reactive protein, PAD and CVD

CRP levels were also associated with incident PAD. However, this association was attenuated after adjustment for cardiovascular risk factors and eventually lost statistical significance when history of CVD was accounted for. When CRP was adjusted for each risk factor individually, smoking and history of CVD were the two variables that had the most pronounced effect on the association between CRP and incident PAD. The importance of smoking as a confounder is not surprising given that it is the most important risk factor for PAD and at the same time is highly correlated with CRP.

Despite the extensive data on CRP and incident CVD from several population studies, its role in peripheral atheroma is not well established. CRP has only been associated with incident PAD in the Physicians Health study which showed that CRP

along with total/HDL cholesterol ratio were the strongest predictors of PAD among several biomarkers assessed in healthy males (Ridker et al 1998c). In comparison to our study, the crude risk ratio of CRP (2.2 top vs. bottom quartile) for incident PAD was little reduced (2.1) in the adjusted analysis. However, this was a nested case control study in which cases and controls were matched for age and smoking and only males were included. Therefore, the adjustment for other risk factors would be expected to have a relatively smaller effect on the estimated risk ratios than in this analysis. Moreover, cross-sectional data have shown that CRP levels are higher in patients with symptomatic PAD (IC or CLI) compared to healthy controls (Cassar et al 2005).

In this study, CRP was also associated with incident CVD (defined as MI or stroke) with a hazard ratio of 2.2 which was very close to the 2-fold relative risk for CHD (top vs. bottom tertile) estimated by the recent meta-analysis of studies on CRP and incident CHD (Danesh et al 2004). Also, the hazard ratio for CVD was slightly higher than the hazard ratio of 2.0 observed between CRP and PAD. Contrary to the PAD analysis, CRP was also significantly associated with incident CVD when cardiovascular risk factors and history of CVD were accounted for. This difference in the effect of CRP on incident PAD and incident CVD (MI or stroke) does not necessarily support a different effect of CRP on different manifestations of the disease. PAD events were considerably fewer than CVD events and this would have itself somewhat influenced the precision of estimated effect sizes and the significant levels. In addition, it should be noted that CRP (and IL-6) were measured in fewer

individuals than the other markers examined in this thesis and this might have also contributed to the precision of the estimated effect sizes and the significant levels.

The causality of the association between CRP and CVD is open to debate given the strong correlation of CRP with traditional cardiovascular risk factors and symptomatic and asymptomatic arterial disease, and the possibility of ‘reverse causality’ (Lowe et al 2005). Interestingly, in this study, CRP was associated with incident CVD even after adjustment for subclinical atherosclerosis measured by the ABI; a result which was also demonstrated by the Cardiovascular Health Study (Cushman et al 2005). This is an important finding which weakens the hypothesis of reverse causality and provides further evidence of a causative role of CRP in atherosclerotic disease. However, adjusting our analysis for multiple measures of subclinical disease in addition to the ABI would add to the validity of this result. Unfortunately other measures of subclinical disease were not available at the baseline examination of the Edinburgh Artery Study and therefore this analysis was not performed.

CRP also showed relative importance as a risk factor for stroke, a finding in support of a meta-analysis of studies with long follow up (>8 years) that showed that the risk for stroke in healthy individuals with the highest quartile of CRP concentrations increased nearly 70% compared to those with the lowest quartile (Kuo et al 2005). However, despite the fact that the association of CRP with stroke has been previously observed by others (Ford et al 2000; Gussekloo et al 2000; Ridker et al 2000a; Ridker et al 1997; Rost et al 2001), its relative importance compared to MI is

controversial. In the 543 healthy males participating in the Physicians' Health Study, CRP was a stronger predictor for MI than for stroke (Ridker et al 1997). In contrast, data from the Honolulu Heart Programme showed that CRP levels had comparable associations with MI and stroke (Curb et al 2003). Above all, the association between increased inflammatory markers and future stroke is of particular interest because it might provide an explanation for the beneficial role of statins in cerebrovascular disease despite the fact that LDL cholesterol is not a strong risk factor for stroke (Ridker 2002).

7.5.3 Role of C-reactive protein in atherosclerosis

The present findings emphasize the tight linkage of CRP to the atherosclerotic processes and may support a hypothesis that CRP directly interacts with atherosclerotic vessels to promote atherosclerotic development and progression. There are many pathways through which CRP may be causally related to atherosclerosis (Armani et al 2005b). For example, CRP has been shown to interact with modified LDL, a fact that may contribute to the regulation of LDL metabolism and foam cell formation in the arterial wall (Rattazzi et al 2003). In addition, CRP could promote the activation of monocytes and induce the expression of adhesion molecules, IL-6 and PAI-1 by endothelial cells further contributing to atherosclerotic development (Pasceri et al 2001). Moreover, CRP can stimulate tissue factor production and activate the complement (Li et al 2004). As stated before, tissue factor is the main stimulus for initiating the coagulation cascade, which may further promote atherosclerosis.

On the other hand, CRP directly affects endothelial function by altering NO bioavailability. NO is a vasodilator synthesized by the endothelial nitric oxide synthase enzyme. In fact, endothelial cells incubated with CRP showed decreased endothelial nitric oxide synthase expression and NO release (Venugopal et al 2002; Verma et al 2002). Two other studies suggested that CRP could also promote activation, proliferation and migration of the smooth muscle cells (Hattori et al 2003; Wang et al 2003).

Further evidence comes from studies that have attempted to localize CRP in the atherosclerotic plaques. An early study confirmed the presence of CRP in human aortic lesion immunohistochemically (Reynolds et al 1987). Since then, studies have replicated this result and have reported the production of CRP from coronary plaques, aneurismal aortas, failed venous coronary bypasses and in femoral atherosclerotic plaques (Vainas et al 2005).

7.6 Interleukin-6

7.6.1 Interleukin-6 and subclinical disease

IL-6 is a pro-inflammatory cytokine which has shown substantial evidence for associations with CHD. This is the first study to examine the predictive value of IL-6 in relation to progression of peripheral atherosclerosis. A significant association between IL-6 and ABI at baseline, ABI change at 5 years, and ABI change at 12 years of follow up in analyses adjusted for conventional risk factors was found.

These results were also confirmed by longitudinal analysis with the use of ABI as a repeated measurement over 3 time points.

Therefore, a strong relationship between IL-6 and progression of atherosclerosis in a series of analyses was reported. Cross-sectional analysis from the Health ABC study (Cesari et al 2003) also showed that for those with IL-6 levels in the highest compared to the lowest tertile, the odds ratio for subclinical CVD (defined by the Angina or IC WHO questionnaire or by $ABI < 0.9$ or by ECG) was 1.58 (95% CI 1.26 to 1.97). On the contrary, in a subsample of the Rotterdam Study (van der Meer et al 2002a), IL-6 levels were not found to be associated with another measure of subclinical atherosclerosis, carotid IMT. This negative result was replicated in a healthy population of 1,111 men and women in the Australian National Heart Foundation Survey (Chapman et al 2004).

IL-6 was also an independent predictor of ABI change at 5 years and at 12 years, when both risk factors and inflammatory markers (CRP, ICAM-1, VCAM-1, and E-selectin) were simultaneously entered as covariates into the model. The independence of IL-6 associations with atherosclerotic disease after adjustments for inflammatory variables was also observed in relation to incident CVD in this thesis and it has been examined in relation to CHD by other investigators. Ridker et al reported that IL-6 remained significantly associated with IHD risk after adjustment for CRP (Ridker et al 2000a). Also, the Quebec Cardiovascular Study replicated this result and showed that IL-6 was associated with long term risk of IHD after adjustment for lipid and non-lipid factors as well as for CRP and fibrinogen. The

authors claimed that the advantage of IL-6 over CRP might be due to the lower biological variability of IL-6, as previously shown (Browning et al 2004). However, since IL-6 is a primary stimulant for acute phase response proteins and also up-regulates adhesion molecules, the present data might explain why acute phase reactants (CRP, fibrinogen) or adhesion molecules have been associated with cardiovascular risk.

IL-6 also showed a greater independent effect on ABI compared to haemostatic markers. Some evidence for an additive effect between IL-6 and the fibrinolytic marker D-dimer was also found. When IL-6 and haemostatic markers were examined in the same model, D-dimer, added to the R^2 value of the model with IL-6 when predicting ABI change. Similarly, subjects with high levels of both D-dimer and IL-6 had a worse disease outcome (greater ABI decline) than those who had one or none of these factors at high levels. This additive effect of D-dimer and IL-6 was also observed in relation to incidence CVD but not to incident PAD. Evidence for an additive effect between these two markers and CHD has been previously reported (Lowe et al 2004b).

IL-6 was the only inflammatory marker that predicted ABI decline after 5 and 12 years of follow up independently of baseline risk factors in subjects without PAD at baseline. Moreover, when the baseline population was divided according to cholesterol levels (above/ below median) or smoking status (current/ex and never smokers), IL-6 was associated with ABI decline with the same magnitude among high or low risk individuals. The same was not true for CRP which had considerably

lower predictive ability when cholesterol levels were decreased. IL-6 has previously shown high correlations with prevalent PAD and CVD and cardiovascular risk factors. The latter subgroup analysis confirmed the original analysis and stressed the fact that IL-6 associations with atherosclerotic progression were not only a consequence of elevated cardiovascular risk factors or prevalent CVD and thus are more likely to be causal.

7.6.2 Interleukin-6, PAD and CVD

In relation to incident PAD, IL-6 was independently associated with clinical PAD events in analysis adjusted for CVD risk factors. However, as with CRP, IL-6 lost significance when the history of CVD at baseline was accounted for, probably due to the high correlation between IL-6 and prevalent CVD. On the other hand, IL-6 was associated with incident CVD (MI or stroke) independently of conventional risk factors and of baseline CVD. In fact, IL-6 showed the greatest hazard ratio (top vs. bottom tertile) for CVD (1.8) compared to other markers studied here and was considerably higher than that estimated for incident PAD (1.5).

Data on IL-6 and PAD are very limited. Several cross-sectional studies have shown that IL-6 is elevated in patients with PAD compared to healthy controls (Brevetti et al 2003;DePalma et al 2003;Erren et al 1999;McDermott et al 2005a;Signorelli et al 2003;Silvestro et al 2003;Yu et al 2004). This is the first study to assess the role of IL-6 in relation to PAD prospectively and the results need cautious interpretation. They may reflect a modest effect of IL-6 on PAD and therefore a possible difference in its predictive ability across diverse disease manifestations. Also, as with CRP,

PAD events were fewer than combined CVD events and this might have itself somewhat influenced the precision of the estimated effect sizes and the significance levels.

The reported associations between IL-6 and CVD are in agreement with previous published studies. In fact, the hazard ratio of IL-6 in analysis adjusted for cardiovascular risk factors was the highest of all other inflammatory markers under study and remained significant after adjustments for other inflammatory markers. IL-6 has been found before to be a stronger predictor of incident CVD compared to other inflammatory markers including CRP or fibrinogen (Luc et al 2003b; Cesari et al 2003). Finally, IL-6 was also associated with incident stroke; a result that has only been reported by the Heath ABC Study (Cesari et al 2003).

In the previous analysis IL-6 was strongly associated with subclinical atherosclerosis and its progression. Notably, here, the associations of IL-6 with incident CVD were independent of subclinical disease measured by the ABI. Therefore, the association between IL-6 and incident CVD is less likely to simply reflect an inflammatory response to early atherosclerotic development and thus more likely to play a causal role in atherosclerotic disease.

Composite score: IL-6, fibrinogen and t-PA

Finally, IL-6 along with fibrinogen and t-PA had the highest hazard ratios for incident CVD. A composite score for elevated concentrations of these markers was calculated. Interestingly, an incremental risk between people with 1, 2 or 3 markers

in the top tertile was found. One possible explanation of this effect could be that these markers might promote atherosclerosis through distinct pathways. Composite scores have previously been reported to add to the ability to assess cardiovascular risk for other biomarkers. Data from the Quebec Cardiovascular Study indicated that an inflammatory score defined as a combination of high IL-6 and fibrinogen levels, in combination with a series of traditional risk factors, may better discriminate incident IHD cases from IHD-free individuals (St-Pierre et al 2005). In addition, in the Health ABC Study a composite summary indicator of inflammation including CRP, TNF- α and IL-6 showed a strong association with cardiovascular events. It must be stressed however that the use of a score based on increased levels of biomarkers needs careful consideration from a clinical viewpoint in terms of cost and time effectiveness.

7.6.3 Role of interleukin-6 in atherosclerosis

Findings of this thesis support a strong association between IL-6 and atherosclerotic progression which is independent of major cardiovascular risk factors and other inflammatory and haemostatic markers. IL-6 is a pro-inflammatory and pro-coagulant cytokine which might have important implications on the atherosclerotic development and progression. In fact, IL-6 induces the production of several inflammatory and haemostatic molecules that have been in turn associated with atherosclerosis and thrombotic complications. Firstly, IL-6 up-regulates the expression of CRP, fibrinogen and other acute phase reactants by the liver. Also, IL-6 increases basal glucose intake, alters insulin sensitivity and induces the expression of adhesion molecules and the secretion of other cytokines by endothelial

cells (Yudkin et al 2000). In addition, it has important pro-coagulant properties promoting platelet production, stimulating coagulants and inhibiting anti-coagulants which might further induce IL-6 production (Kerr et al 2001). Finally, IL-6 may also be associated with plaque stability through its role in extracellular matrix deposition and reorganization (Galis et al 1994; Grote et al 2003; Schieffer et al 2004; Solis-Herruzo et al 1999).

IL-6 was also found within human atherosclerotic plaques in different vascular beds by several investigators further suggesting a vital and potentially causative role of this molecule in atherosclerotic disease (Kaneko et al 1997; Rus et al 1996; Salomon et al 1992; Seino et al 1994). Scheiffer showed that IL-6 is expressed particularly at the shoulder of the atherosclerotic plaque (Schieffer et al 2000). In a later report, Scheiffer (Schieffer et al 2004) showed a direct link between IL-6 and plaque decomposition in mice models. In this study, a deficiency in IL-6 enhanced atherosclerotic plaque development and was associated with increased lipids levels (Schieffer et al 2004).

7.7 Adhesion molecules

7.7.1 Adhesion molecules and subclinical disease

The relationship between adhesion molecules and the extent of atherosclerosis is very little studied whereas existing reports have shown conflicting results. In the present analysis, ICAM-1 showed significant associations with progressive peripheral atherosclerosis after 5 and after 12 years of follow up. Also, the

associations at 12 years were independent of cardiovascular risk factors and baseline CVD. Additionally, ICAM-1 was a significant predictor of ABI change at 5 and 12 years of follow up in subjects without PAD at baseline (baseline ABI and age adjusted analyses). Results on ICAM-1 and progression of atherosclerosis were also confirmed by repeated measures analysis. VCAM-1 and E-selectin showed no significant associations with ABI change in any analysis and indeed they were not even associated with baseline ABI.

Previous evidence showed that ICAM-1 and VCAM-1 were independently associated with ABI among 88 subjects (Iwashima et al 2005). Also, strong correlations between carotid IMT and plasma ICAM-1 and VCAM-1 have also been reported (Rohde et al 1998). In addition, ICAM-1 was independently associated with the risk of having at least one carotid plaque and with the risk of having at least one femoral plaque but not with IMT in another study (Bongard et al 2002). Moreover, the present study supports previous evidence for a different role between ICAM-1 and VCAM-1 according to the stage of the disease. In fact, studies have shown that VCAM-1 is associated with PAD progression in subjects with pre-existing disease, but ICAM-1 predicts disease development in healthy populations as found in this cohort. Finally, our null data for E-selectin is in accordance with previous studies, which did not find any association between E-selectin levels and PAD severity or PAD location (De Caterina et al 1997; Blann et al 1997; Blann et al 2000).

7.7.2 Adhesion molecules, PAD and CVD

Plasma levels of ICAM-1, but not of VCAM-1, were strongly associated with incident PAD and retained statistical significance after adjustment for traditional risk factors. This is in accordance with one prospective study which had examined the association between ICAM-1 and VCAM-1 with symptomatic PAD (Pradhan et al 2002a). The authors concluded that ICAM-1, and not VCAM-1, was associated with more than a 3 fold increase in the risk of developing symptomatic PAD in analysis adjusted for cardiovascular risk factors (top vs. bottom quartile) (Pradhan et al 2002a). In addition, in the present analysis the hazard ratio of ICAM-1 in the adjusted analysis was 1.8 (top vs. bottom tertile) which was little changed when analysis was further adjusted for levels of other inflammatory markers. No evidence was found for an association of E-selectin and PAD development which is in agreement with previously published negative results for the presence and progression of peripheral atherosclerosis (Blann et al 1998;De Caterina et al 1997;Peter et al 1997).

In contrast to PAD analysis, ICAM-1 showed significant associations with incident CVD only when adjusted for age and sex and lost significance in the multivariable analysis. Other studies have also failed to show significant associations between ICAM-1 and incident CVD in the multivariable analysis (Ridker et al 2000a; Jenny et al 2006). Moreover, the combine meta-analysis of 5 studies on ICAM-1 and CHD has yielded a modest odds ratio of 1.4 (top vs. bottom tertile) (Malik et al 2001). A potentially favorable role of ICAM-1 in peripheral rather than coronary or cerebrovascular atherosclerosis could be argued from results reported here. ICAM-1

showed strong associations with ABI decline and with incident PAD but not with incident CVD defined as MI or stroke. However, since there is no biological mechanism that could explain this importance of ICAM-1 in the peripheral vascular bed, its relative value needs replication from future studies.

Data on VCAM-1 and E-selectin are generally negative for associations with incident CVD and therefore they are in line with results reported here (Malik et al 2001). Interestingly, E-selectin had a significant hazard ratio when incident stroke was examined separately and retained significant associations in the multivariable analysis. Since there are no other published data on E-selectin and stroke, a preferential effect of E-selectin on stroke events only needs cautious consideration and validation from future studies.

7.7.3 Role of adhesion molecules in atherosclerosis

The aforementioned analysis showed a strong association between ICAM-1 and the progression and incidence of peripheral atherosclerosis and provided further support for a role of adhesion molecules in atherosclerotic development. In fact, adhesion molecules are thought to contribute to the earliest stage of atherosclerotic disease by facilitating leukocyte adhesion to the endothelium and their migration to the intima. E-selectin is thought to play a pivotal role in rolling and tethering of the leukocytes in the arterial wall whereas ICAM-1 and VCAM-1 are thought to participate in the later stages of firm adhesion and transmigration of leukocytes across the endothelium (Hope 2003).

In addition, expression of adhesion molecules has been found in atherosclerotic plaques (Huo et al 2001). E-selectin expression by endothelial cells was shown on the surface of fibrous and lipid containing plaques (Davies et al 1993). However, the expression of E-selectin was not found in atherosclerotic plaques of rabbits or mice (Iiyama et al 1999). Increased expression of ICAM-1 was also observed on the surface of human atherosclerotic plaques and on the surface of lesion-prone areas in rabbits or mice (Iiyama et al 1999;Poston et al 1992). Finally, VCAM-1 was expressed on the endothelial cells in fibrous and lipid containing plaques (Davies et al 1993;Iiyama et al 1999).

7.8 Fibrinogen

7.8.1 Fibrinogen and subclinical disease

Fibrinogen is the most extensively studied haemostatic marker as a risk factor for CVD. In this analysis, fibrinogen was associated with progression of peripheral atherosclerosis as evaluated by changes of ABI after 12 years. This association was independent of cardiovascular risk factors and also of other haemostatic factors studied here. At 5 years, no significant associations were found between fibrinogen levels and ABI reduction probably due to small power of this analysis since the ABI change after 5 years was much smaller compared to that over 12 years.

The association between elevated fibrinogen and subclinical atherosclerotic disease has been previously examined in cross-sectional studies only. In the Cardiovascular Health Study, fibrinogen was associated with subclinical atherosclerosis measured by

a variety of methods including electrocardiography, echocardiography, carotid ultrasound and the ABI (Tracy et al 1995). Other studies have confirmed the association between fibrinogen and asymptomatic atherosclerosis measured by the ABI (McDermott et al 2003; Philipp et al 1997). In 693 hypercholesterolemic men, fibrinogen was also associated with the presence of carotid, femoral and aortic plaque (Levenson et al 1997). Moreover, fibrinogen has been associated with carotid IMT and with carotid plaque formation independently of cardiovascular risk factors (Chapman et al 2004; De Maat et al 2003; Martinez-Vila et al 2003). The ARIC study has also evaluated the relationship between plasma fibrinogen and progression of carotid atherosclerosis measured by IMT change. A significant relationship between fibrinogen and IMT change has only been observed in the univariable analysis (Chambless et al 2002).

The present analysis showed a significant association between fibrinogen levels and progression of peripheral atherosclerosis over 12 years. This effect of fibrinogen was limited in the absence of cardiovascular risk factors cholesterol and smoking. Also, the individual effect of fibrinogen on ABI decline was smaller than that estimated for CRP or IL-6 whereas adjustments for any of these markers reduced the reported associations to non-significance for fibrinogen. These results highlight an important role of fibrinogen in atherosclerotic progression but on the same time suggest that these associations are to some extent due to interrelationships of fibrinogen with cardiovascular risk factors and with other inflammatory markers.

Finally, fibrinogen and D-dimer were the only haemostatic markers examined in this thesis that showed associations with peripheral atherosclerotic progression. In fact, other haemostatic markers were not even associated with baseline ABI. Fibrinogen and D-dimer are acute phase reactants and therefore these results might demonstrate a primary role of inflammation rather than of haemostasis in atherosclerotic development and progression.

7.8.2 Fibrinogen, PAD and CVD

Fibrinogen was associated with PAD independently of conventional risk factors and history of CVD. Its hazard ratio of 2.2 for subjects in the top vs. the bottom tertile of fibrinogen at baseline was higher than any of the other markers studied here.

Fibrinogen was also significantly associated with incident PAD in the absence of major PAD risk factors including smoking and cholesterol levels. The association between fibrinogen and PAD has been confirmed by many cross-sectional studies (Fabsitz et al 1999;McDermott et al 2003;McDermott et al 2005a;Meijer et al 2000;Selvin et al 2004;Smith et al 1993;Wildman et al 2005;Zheng et al 2005).

However, evidence from prospective studies is very limited. The prognostic value of fibrinogen is illustrated by the 5 year follow up of the Edinburgh Artery Study, in which median fibrinogen levels were higher in patients who later went on to develop PAD (Smith et al 2000b). These results were confirmed by the Physicians' Health Study (Ridker et al 2001).

Apart from PAD, fibrinogen also presented significant associations with incident CVD independently of cardiovascular risk factors and incident CVD. This is in

accordance with the recent individual participant meta-analysis which found moderately strong associations between usual plasma fibrinogen level and the risks of CHD, stroke, other vascular mortality, and nonvascular mortality in a wide range of circumstances in healthy middle-aged adults (Danesh et al 2005). In the present analysis, fibrinogen also had significant hazard ratios for both MI and stroke and retained significant associations after adjusting for subclinical atherosclerosis. The latter has also been observed for CRP and IL-6 and is of particular importance because it implies that elevated fibrinogen levels are more likely to be causally associated with CVD than to simply reflect the result of CVD risk factors and subclinical or clinical atherosclerotic disease.

7.8.3 Role of fibrinogen in atherosclerosis

This thesis has provided evidence that fibrinogen might not only be a marker but also might have a role in the pathophysiology of atherosclerotic disease. In fact, several roles of fibrinogen in atherosclerotic development and progression have been proposed. For example, fibrinogen binds to ICAM-1 on endothelial cells which may lead to the release of vasoactive mediators, enhanced monocyte- endothelial cell interaction and increased platelet adhesiveness (Hicks et al 1996;Kamath et al 2003). The binding of fibrinogen on ICAM-1 also up-regulates its gene expression, which further promotes the adhesion of leukocytes, platelets, and macrophages to endothelial cells (Kamath et al 2003).

Also, fibrinogen has been shown to promote smooth muscle cell proliferation and migration (Kamath et al 2003). Fibrinogen may also play a role in foam cell

formation by facilitating the transfer of cholesterol molecules from platelets to macrophages (Koenig 2003). It may also contribute to platelet aggregation and thrombus formation by binding to GPIIb/IIIa receptor and it also affects thrombus size, structure and deformability (Koenig 2003). In addition, fibrinogen is a major determinant of plasma viscosity and erythrocyte aggregation and, therefore, affects blood viscosity which has in turn shown associations with CVD (Koenig et al 1992). Finally, fibrinogen has been shown to accumulate inside atherosclerotic plaques (Bini et al 1989).

7.9 D-dimer and tissue plasminogen activator

7.9.1 D-dimer, tissue plasminogen activator and subclinical disease

The aforementioned analysis showed significant associations of D-dimer with atherosclerotic progression, independently of other haemostatic factors and conventional risk factors. However, D-dimer failed to retain significant associations with ABI change when results were adjusted for IL-6 levels. D-dimer has shown an additive effect with IL-6 levels as was already discussed in the IL-6 section. In addition, t-PA showed no significant associations with ABI decline even in the univariable analysis and was only weakly associated with baseline ABI.

D-dimer and t-PA have been extensively studied in relation to CHD but epidemiological research on asymptomatic atherosclerotic disease is scarce. In accord with our results, D-dimer, but not t-PA, has been independently associated with ABI in two cross-sectional studies (Cushman et al 1996;McDermott et al 2003).

Moreover, neither of them has shown evidence for associations with IMT in three other studies (De Maat et al 2003; Lee et al 1998; Salomaa et al 1995). Also, in patients with symptomatic PAD, D-dimer was associated with the occurrence of myocardial infarction but not with progression of atherosclerosis assessed by changes in ABI or carotid duplex scanning progression (Musicant et al 2006).

7.9.2 D-dimer, tissue plasminogen activator, PAD and CVD

As in previous analysis, D-dimer but not t-PA was significantly associated with incident PAD independently of cardiovascular risk factors and history of CVD disease. In addition, D-dimer showed a dose response relationship with disease severity. The relationship between these fibrinolytic markers and PAD is undetermined. In previous analysis of the Edinburgh Artery Study, t-PA and D-dimer were not associated with incident PAD after 5 years of follow up. However, both plasma markers have shown higher levels with the presence and increasing severity of PAD in cross-sectional analyses (Cassar et al 2005; Killewich et al 1998; Smith et al 1995; Unlu et al 2006).

Both these markers were associated with incident CVD independently of CVD risk factors, history of CVD and subclinical disease and their estimated hazard ratios are in agreement with those yielded from meta-analysis of prospective studies on CHD (Danesh et al 2001; Lowe et al 2004a). However, the association of D-dimer was marginal and much weaker than that observed for fibrinogen, CRP or IL-6. On the other hand, t-PA had a relatively high association with incident CVD in the multivariable model but this was considerably decreased when individuals with

history of CVD were excluded from the analysis. Therefore, t-PA might be especially important in people with pre-existing disease as previous studies on patients with angina pectoris have shown (Held et al 1997; Juhan-Vague et al 1996; Smith et al 2000a). Finally, results showing associations of t-PA with incident MI and stroke but not with the presence and progression of asymptomatic atherosclerosis might reflect an importance of this molecule in later disease stages (e.g. plaque rupture) rather than in the early (asymptomatic) disease stage.

7.9.3 Role of D-dimer and tissue plasminogen activator in atherosclerosis

Results from the aforementioned analysis showed a modest effect of D-dimer and t-PA in atherosclerotic progression and its complications. In particular, D-dimer was associated with several manifestations of atherosclerosis (ABI decline, PAD and CVD) whereas t-PA showed no evidence for associations with asymptomatic and progressive atherosclerosis but was associated with incident CVD defined as MI or stroke. Levels of t-PA and D-dimer reflect plasmin and fibrin formation, respectively. Von Rokitansky, in 1852, first suggested that the persistence of fibrin deposits at the site of injury may promote the formation of an atherosclerotic lesion (Robbie et al 2001). According to this hypothesis these molecules might reflect the contribution of intravascular fibrin to atherogenesis as well as to thrombogenesis (Lowe et al 1999). There is also evidence that D-dimer within the evolving plaque influences atherogenesis through several other mechanisms including modulation of endothelial cell permeability, and stimulation of migration and proliferation of smooth muscle cells (Rabbani et al 1994). D-dimer is also chemotactic for

monocytes and provides a surface for the accumulation of LDL cholesterol (Thompson et al 1989).

The role of the fibrinolytic system in the development of the atherosclerotic plaque was further supported with reports demonstrating the deposition of fibrin and its degradation products in atherosclerotic arterial walls. In one study (Raghunath et al 1995) on human coronary arteries, t-PA tended to be more abundant in the intima of atherosclerotic arteries, with the intima/media ratio of t-PA being significantly higher in these atherosclerotic arteries. Similarly, smooth muscle cells located in the media showed a strong staining for t-PA mRNA and antigen, suggesting a constant t-PA production (Lupu et al 1995). Moreover, the main inhibitor of fibrinolysis PAI-1 is also more abundantly expressed in tissues of patients with atherosclerosis (Spronk et al 2004). D-dimer has also shown increased concentrations in atherosclerotic plaques and has also shown increased levels with increasing severity of atherosclerotic plaques (Bini et al 1989).

7.10 Other haemostatic markers

There was very little evidence from results presented in this thesis for associations between vWF, factor VII, F1+2 or FpA and PAD. In fact, none of these markers was associated with the progression of peripheral atherosclerosis and incident PAD whereas only vWF was associated, albeit weakly, with incident CVD.

The literature review on the introduction of this thesis showed that factor VII, F1+2 and FpA have received little attention in the literature as potential risk factors for

CVD and that existing evidence for associations is very weak. vWF has been more extensively studied and a recent meta-analysis shows that relative risks of CHD associated with increased vWF were relatively modest (Whincup et al 2002). In relation to PAD, in support with findings presented here, previous investigators (Folsom et al 1993; McDermott et al 1993; McDermott et al 2003; Philipp et al 1997; Tracy et al 1995), as well as previous reports of the Edinburgh Artery Study (Lee et al 1998; Lowe et al 1993; Smith et al 2000b), have also failed to find independent associations between these markers and ABI, PAD development or measures of asymptomatic disease such as the IMT.

These negative results however need cautious interpretation. The lack of associations presented here and in other reports does not necessarily mean that these molecules are not involved in atherosclerotic disease and its progression. On the other hand, lack of associations might be due to the pre-analytical and analytical properties of these markers which may not be adequate for their measurement in epidemiological studies or clinical routine. For example, FpA was highly variable (range 1.0-50.2 ng/ml) and its assay had limited sensitivity because 38% of the population had levels <1ng/ml.

7.11 Inflammation, haemostasis and atherosclerotic disease

The primary aim of this analysis was to examine associations between a number of markers and the progression of atherosclerotic disease in order to identify possible mechanisms involved in the aetiology, development and progression of disease.

Results have confirmed an association between the activation of these two mechanisms and atherosclerosis. CRP, IL-6 and fibrinogen have shown associations with peripheral atherosclerotic progression and incident PAD, MI and stroke. To a lesser extent, the same was true for D-dimer and ICAM-1.

Several of these markers may be used as a tool to better identify individuals at high risk of developing a cardiovascular event. However, the usefulness of these markers in risk prediction models and clinical practice was beyond the scope of this thesis and therefore was not examined. The clinical value of each marker should be judged both upon its incremental value above conventional risk factors and its assay/measurement features.

Results reported here are in favour of a fundamental role of coagulation, fibrinolysis, and inflammation in the atherosclerotic process. Biological markers reflecting the activation of these mechanisms were associated with several phases of the atherosclerotic process, from lesion initiation through to progression and, ultimately, the thrombotic complications of atherosclerosis. For many of these markers, associations between their levels and an increased risk of cardiovascular events have been repeatedly examined before. However, this is the first time that associations between their levels and the progression of atheroma have been examined prospectively in the general population. Our findings showing that CRP, IL-6, fibrinogen and to a lesser extent D-dimer and ICAM-1 are associated with the progression of atherosclerotic disease have important implications. In fact, these findings are highlighting a role of these molecules in the early atherosclerotic process

and not only in vulnerable atherosclerotic plaques, as it has often been hypothesised. Moreover, these markers may be useful in assessing the progression of asymptomatic disease in individuals who are seemingly healthy but are at high risk of developing a cardiovascular event.

Also, the associations reported here were independent of conventional risk factors, which supports a possible causal role of inflammation and haemostasis in atherosclerotic disease. The same conclusions can be drawn from the fact that the same markers were associated with incident CVD independently of asymptomatic atherosclerosis at baseline.

Moreover, this thesis has examined the relative value of several biomarkers in the same cohort and has thus had the ability to compare their effects and their associations with cardiovascular disease. Most importantly, some of these markers have shown evidence for an additive effect on disease progression. It is important to note that the biological pathways of inflammation and haemostasis are not independent but rather they are interconnected (Esmon 2004). This is also reflected by the high correlation coefficients found between various markers. It is therefore essential to focus not just on the individual pathways but also on the ways that these molecules interact. This will then enable the design of new therapeutic and preventive strategies that interrupt pathologic activities, such as the initiation and progression of atheroma, without affecting homologous physiologic activities, such as the acute phase response or wound repair, which often involve the same mediators (Tracy 2003).

Chapter 8

8 Conclusions and Recommendations

In this chapter, the principal conclusions are summarized and the recommendations for further research are listed.

8.1 Conclusions

1. Atherosclerotic progression was measured by the change in ABI after 5 and after 12 years of follow up. The mean ABI change was -0.04 and -0.06 at 5 and 12 years respectively. This decline was statistically significant and reflected a progression of atherosclerotic disease in our population over the years.
2. Risk factors for progression of subclinical PAD were similar to those for clinical PAD and included age, male sex, smoking, total/HDL cholesterol, hypertension and pre-existing CVD. Prevalence of diabetes was higher among those who developed PAD or whose ABI declined but it failed to reach statistical significance.
3. CRP, IL-6 and ICAM-1 were associated with ABI decline independently of conventional risk factors and baseline CVD. These findings suggest that these molecules are molecular markers associated with atherosclerosis and its progression. IL-6 showed more consistent results and stronger independent predictive value than other inflammatory markers.
4. Fibrinogen and D-dimer, but no other haemostatic markers, were associated with ABI decline independently of conventional risk factors and baseline CVD. Since D-dimer and fibrinogen are acute phase reactants, these data support the

hypothesis that inflammation is more related to atherosclerosis than is hypercoagulation. Moreover, associations between fibrinogen or D-dimer and ABI change were no longer significant when analyses were adjusted for either CRP or IL-6. There was also some evidence for an additive role of interleukin-6 and D-dimer in progressive atherosclerosis.

5. IL-6 showed the greatest independent effect of all markers studied here and had significant associations with ABI change event after adjustments for inflammatory and for haemostatic markers. Elevated levels of IL-6 might reflect the extensive interaction between inflammation and coagulation which, after repeated cycles, leads to atherosclerotic progression.
6. CRP and IL-6 showed modest associations with incident symptomatic PAD which were no longer statistically significant in the multivariable analysis. These results may reflect a modest effect of these markers on PAD and therefore a possible difference in their predictive ability across diverse disease manifestations. On the other hand, these findings might be in support of recent evidence from other studies that have also demonstrated a modest effect of CRP on CVD prediction (Danesh et al 2004; Lawlor et al 2005). Finally, CRP and IL-6 were measured in fewer individuals at baseline than other markers studied here and this might have influenced the effect sizes and the significant levels.
7. ICAM-1 showed strong associations with incident PAD and retained statistical significance after adjustment for traditional risk factors. ICAM-1 has also been associated with progression of asymptomatic PAD and therefore might be an important marker for peripheral atherosclerosis.

8. Levels of CRP, IL-6, fibrinogen, D-dimer and t-PA had significant and independent hazard ratios associated with incident CVD even when analysis was adjusted for subclinical disease at baseline (measured by the ABI). These markers, with the exception of t-PA, have also been associated with asymptomatic atherosclerotic disease which may further induce their production. These results show that their association with future CVD was independent of asymptomatic atherosclerosis and thus they are less likely to simply reflect an inflammatory or haemostatic response to early atherosclerotic development ('reverse causality').
9. IL-6, fibrinogen and t-PA had the strongest independent effect on development of CVD compared to other factors in this study, with hazard ratios between their top and bottom tertiles greater than 1.8. An incremental risk between people with 1, 2 or 3 markers in the top tertile was found. One possible explanation of this effect could be that these markers might promote atherothrombosis through distinct pathways.
10. CRP and E-selectin showed a relatively stronger associations with stroke than with MI. Increased inflammatory markers associated with future stroke might reflect a larger role of inflammation in stroke rather than in MI, and might provide an explanation for the beneficial role of statins in cerebrovascular disease despite the fact that LDL cholesterol is not a strong risk factor for stroke.
11. Other markers investigated in this thesis including VCAM-1, factor VII, FpA, F1+2 showed no evidence for associations with ABI decline, incident PAD or CVD. E-selectin was associated with incident stroke only, whereas vWF showed

modest associations with incident CVD. These results suggest that these molecules are not good markers of atherosclerotic disease.

8.2 Recommendations

1. The ABI change was used to assess asymptomatic atherosclerotic disease in the study population over 5 and 12 years of follow up. The progression of asymptomatic disease prospectively is very little studied in the literature and further studies are needed to investigate the progression of disease over time. Other markers of subclinical disease such as the IMT need to be examined as well. Most importantly, the simultaneous study of several non-invasive tests would be very informative on the progression of systemic atherosclerosis.
2. In this thesis, levels of inflammatory and haemostatic markers have been associated with ABI decline prospectively. Associations between these markers and other measures of asymptomatic disease need to be examined in order to validate conclusions drawn here.
3. In the Edinburgh Artery Study, inflammatory and haemostatic markers were measured at baseline examination. Further studies should investigate longitudinal measurements of these markers and the effect of the changes of their levels on atherosclerotic progression and cardiovascular risk.
4. Further studies are required to determine whether the relationships between CRP, IL-6, fibrinogen, D-dimer and ICAM-1 with the development of PAD and progression of peripheral atherosclerosis are likely to be causal. Randomized control trials are needed to test this hypothesis by assessing the effects of agents which lower markers' levels on the risk of CVD, PAD or on the progression of

atherosclerosis. To be informative the agents need to be specific and to lower only the markers under investigation.

5. Also, genetic epidemiology and Mendelian randomization studies would be very informative. In this design, genetic variants serve as unconfounded markers of exposures and better allow the test of causality. Current studies on inflammatory/ haemostatic genotypes are inconclusive and larger cohorts are needed to draw definitive conclusions using this approach (Smith et al 2006).
6. Moreover, the interaction between the inflammatory and haemostatic markers needs further investigation in order to understand the complex association between inflammation and haemostasis and thus design relevant pharmaceutical agents for cardiovascular risk prevention. Some evidence for an additive effect between different markers was shown here. The use of a predictive 'score' according to the number of elevated inflammatory/ haemostatic markers needs further investigation.
7. The laboratory measurement of these markers requires further research so that the tests might be more relevant to epidemiological research and clinical practice. Low analytical variability is fundamental. At the same time, the assays need to be standardized, inexpensive and with high sensitivity and specificity.
8. The utility of novel risk markers for screening and risk prediction should be judged upon their incremental value over and above conventional cardiovascular risk factors. Prospective studies should examine potential additional utility in the context of existing risk factors by the calibration of models with the inflammatory/ haemostatic marker and by comparing the areas under receiver-

operating characteristic curves (AUCs) or c-statistics for risk scores calculated with and without the inflammatory/ haemostatic marker.

9. Until the value of these potential risk factors in atherosclerotic disease is evaluated, attention in clinical practice should be given to modifying conventional cardiovascular risk factors associated with PAD and its progression, as cigarette smoking and plasma cholesterol levels.

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APPENDIX

Appendix I

WHO Angina/ IC questionnaire

1. Chest Pain

Yes	No
<input type="checkbox"/>	<input type="checkbox"/>

(a) Do you ever get pain discomfort in your chest?

IF NO, PROCEED TO QUESTION 2

(b) Do you get pain discomfort when you walk uphill or hurry?	<input type="checkbox"/>	<input type="checkbox"/>
---	--------------------------	--------------------------

IF NO, PROCEED TO QUESTION 2

(c) Do you get pain discomfort when you walk at an ordinary pace on the level?	<input type="checkbox"/>	<input type="checkbox"/>
--	--------------------------	--------------------------

(d) When you get any pain or discomfort in your chest what you do?

Tick one

Stop	<input type="checkbox"/>
------	--------------------------

Slow down	<input type="checkbox"/>
-----------	--------------------------

Continue at same pace	<input type="checkbox"/>
-----------------------	--------------------------

Yes	No
<input type="checkbox"/>	<input type="checkbox"/>

(e) Does it go away when you stand still or sit down?

(f) How soon?

Tick one

10 minutes or less	<input type="checkbox"/>
--------------------	--------------------------

More than 10 minutes	<input type="checkbox"/>
----------------------	--------------------------

(g) Where do you get this pain or discomfort? Mark the place(s) with an 'X' on the diagram

	Yes	No
2. (a) Have you ever had a severe pain across the front of your chest lasting for half an hour?	<input type="checkbox"/>	<input type="checkbox"/>

(b) What was the cause?
.....

FOR OFFICE USE ONLY A:

GRADE:

MI:

2. Leg Pain

Yes	No
<input type="checkbox"/>	<input type="checkbox"/>

(a) Do you get a pain in either leg on walking?

IF NO, GO TO QUESTION 2

(b) Does this pain ever begin when you are standing still or sitting?	<input type="checkbox"/>	<input type="checkbox"/>
---	--------------------------	--------------------------

(c) Do you get this pain in your calf (or calves)?	<input type="checkbox"/>	<input type="checkbox"/>
--	--------------------------	--------------------------

(d) Do you get it when you walk uphill or hurry?	<input type="checkbox"/>	<input type="checkbox"/>
--	--------------------------	--------------------------

(e) Do you get it when you walk at an ordinary pace on the level?	<input type="checkbox"/>	<input type="checkbox"/>
---	--------------------------	--------------------------

(f) Does the pain ever disappear when you are still walking?	<input type="checkbox"/>	<input type="checkbox"/>
--	--------------------------	--------------------------

(g) What do you do if you get it when you are walking?

Tick one

a. Stop	<input type="checkbox"/>
---------	--------------------------

b. Slow down	<input type="checkbox"/>
--------------	--------------------------

c. Continue at same pace	<input type="checkbox"/>
--------------------------	--------------------------

(h) What happens to it if you stand still?

Tick one

a. Usually continuous for more than 10 minutes	<input type="checkbox"/>
--	--------------------------

b. Usually disappears in more than 10 minutes	<input type="checkbox"/>
---	--------------------------

	Yes	No
(i) Have you ever had surgery on the arteries of your legs other than varicose veins?	<input type="checkbox"/>	<input type="checkbox"/>

Please specify:.....

(j) Have you evr had surgery to remove

a. Toes	<input type="checkbox"/>	<input type="checkbox"/>
---------	--------------------------	--------------------------

b. Leg below the knee	<input type="checkbox"/>	<input type="checkbox"/>
-----------------------	--------------------------	--------------------------

c. Leg above the knee	<input type="checkbox"/>	<input type="checkbox"/>
-----------------------	--------------------------	--------------------------

FOR OFFICE USE ONLY

Appendix II

Cover letter and annual questionnaire to subjects of the Edinburgh Artery Study

Date

Reference number of subject

Name and address of subject

Dear Name of Subject,

Many thanks for your continuing participation in the Edinburgh Artery Study. We now enclose this year's questionnaire to find out if you have developed any of the listed medical conditions for the **first time** since we last contacted you. Please note that we are **not** asking you to attend for a medical examination.

This information is helping our research team find out more about why people get heart attacks, strokes and artery disease. Since it is very important that we find out about the health of everyone in the study, we should like to hear from you even if you have been perfectly well during the last year.

We should therefore be most grateful if you would take a few minutes of your time to complete the enclosed questionnaire, and return it in the prepaid envelope.

Many thanks for your continuing co-operation.

With best wishes.

Yours sincerely,

Joanna Tzoulaki
Study Co-ordinator

Professor FGR Fowkes
Study Director

EDINBURGH ARTERY STUDY

2005 QUESTIONNAIRE

Please complete and return in the prepaid envelope

PLEASE TICK

Yes No

1. Have you changed your address in the last year?

☐☐

If yes - New address :

.....

2. Have you changed your GP in the last year?

☐☐

If yes - New GP name :

Address:.....

3. Have you experienced any of the following for the **first time** in the last year?

Yes No Not Sure

- (i) Severe pain in your chest?

☐☐☐

(excluding any pain which was treated as an infection)

- (ii) Heart attack?

☐☐☐

- (iii) Angina?

☐☐☐

- (iv) Stroke?

☐☐☐

- (v) Pain in either leg below the knee when walking?

☐☐☐

(excluding any pain due to varicose veins or arthritis)

- (vi) Blood clot or hardening of the arteries in either leg?

☐☐☐

4. Have you attended your GP with any of the above?

☐☐☐

5. Have you attended hospital as an outpatient with any of the above?

☐☐☐

If yes, which hospital?

.....

Date of attendance?

.....

6. Have you been admitted to hospital with any of the of above?

☐☐☐

If yes, which hospital?

.....

Date of admission?

☐☐☐

.....

6. Do you take aspirin daily?

MANY THANKS FOR ANSWERING THESE QUESTIONS